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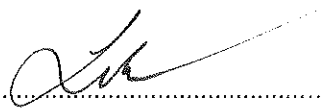
Student's signature:  Date: 20.09.2013

# The Human Vascular Vitamin D Hormonal System – Expression and Regulation

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Thesis submitted for the degree of

*Doctor of Philosophy*

May 2013

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Dedykuję ten doktorat moim najukochańszym rodzicom –

*Krystynie i Andrzejowi Lubczańskim,*

za ich nieskończoną miłość, wiarę i inspirację

I would like to dedicate this thesis to my parents –

Krystyna and Andrzej Lubczanscy

for their immense Love, Faith and Inspiration

# Acknowledgements

I count myself fortunate to have had the opportunity to work in such a friendly, entertaining and dynamic environment. Dr Daniel Zehnder and Dr Rosemary Bland have been phenomenal supervisors and I truly appreciate all the assistance they have provided. Daniel and Rose have been great to work with, whether in the lab or during our lengthy lab meetings when we used to discuss not only science but philosophy, religion, travel, keeping bees for home-made honey and all other sorts of useful DIY also come to mind. Special mention goes to Dr Guerman Molostvov for his wealth of knowledge of everything technique and SMC-related, also for being such an amazing discussion companion and for his love of classical music, which used to make our lab work so pleasurable. Also, I would like to thank Guerman for sharing with me some of the human artery real-time RT-PCR data. I would like to thank Sean James for his assistance with preparation of arterial sections for immunohistochemistry and for his immense optimism, and great sense of humor – staining would never be the same without you! Members of the group Stephen, Matt, Sunil, Dave and Ken have been great to work with, fun to socialize with and have provided many fond memories and with all my heart I wish them all the best for the future.

The time spent in CSRL and exposure to the cutting edge science and scientists, attending variety of talks and seminars, meeting patients have all been highlights of my Ph.D. I would like to thank Dr Ming Xue for all his brilliant technical tips, and for always being there when something needed urgent attention.

I thank Warwick Medical School for funding my Ph.D. I also would like to thank Warwick Medical School, Abbott Laboratories and the Physiological Society for providing me with funds to attend international conferences. I would like to apologise to Rose for almost giving her a heart-attack in Edinburgh, but I hope that I have redeemed myself by receiving an unexpected award for one of the best talks of the meeting.

Having Zehra, Fozia, Florence, Yi, Fang, Tim, Warunee, Phil, Ciara, Becky and Dre around was the assurance of entertaining time in the lab. We all knew what the Eureka moment felt like and we also could empathise with each other when weeks or

months of experiments did not work. Zehra thank you for all your patience, love, understanding, but also for introducing me to classics of Bollywood cinematography and for treating me with your most divine curries. I would like to thank Dre for not giving up on me even though, lab work always came first and I never found time to go kiting. However, the summer of playing tennis in the sun after long hours in the lab will never be forgotten. I would like to thank my other amazing friends Tessa and Penny whom I lived with during my final year of Ph.D. for creating a warm home.

Most of this thesis has been written in Hamburg, after work. My manager Dr Michaela Marose and my friend and work colleague Dr Mujtaba Zaka have been a great source of positive energy, which helped me to power through.

Finally, I must thank Edd for the encouragement, for his patience, understanding and support. I must thank my family: my brothers and sister, Wojtek, Paweł and Basia, and my parents Krystyna and Andrzej, for putting up with me for the past three and a half years – if it was not for them I would never be as proud of this thesis as I am.

I truly hope that this work can help others to find the rest of missing puzzles required for designing the ultimate treatment for CKD related vascular complications.

# Declaration

*I declare that all the work presented in this thesis, except where specifically stated, was original research performed by myself under the supervision of Dr Daniel Zehnder and Dr Rosemary Bland. None of this work has been previously submitted for any other degree. All sources have been acknowledged by means of references.*

# Abstract

**Background:** Patients suffering from chronic kidney disease (CKD) are at high risk of cardiovascular related premature death. In addition, traditional strategies for preventing cardiovascular disease (CVD) in patients with normal kidney function are less successful in patients with CKD. Vascular calcification (VC) and cardiac hypertrophy are common consequences. Recent evidence suggests that activation of the vitamin D receptor (VDR) is protective and is associated with significantly better survival in end stage renal disease (ESRD).
Vascular smooth muscle cells (VSMCs) play a key role in the process of CKD-related arteriopathy and are capable of phenotype transformation into chondro/osteoblast-like cells, which promotes the deleterious vascular effects, including calcification.
Although the kidney is the main site of $1,25(OH)_2D$ synthesis, some other non-renal tissues also express, VDR, 25-hydroxyvitamin D 1α-hydroxyalse (1α-OHase) an enzyme responsible for the synthesis of $1,25(OH)_2D$, and $1,25(OH)_2D$ 24-hydroxylase (24-OHase), an enzyme responsible for the catabolism of $1,25(OH)_2D$. Extra-renal $1,25(OH)_2D$ can affect a multitude of pathways in an autocrine/paracrine way, without the involvement of the endocrine system.

**Aims:** This project aimed to investigate the role of autocrine vitamin D signalling in human VSMCs using both arterial tissue from ESRD and non-ESRD patients and primary cultures of human aortic smooth muscle cells (HAoSMCs). Specific aims were to characterise the expression and functionality of vitamin D system in vasculature as well as to examine the effect of classical regulators of the endocrine vitamin D system – $1,25(OH)_2D_3$, calcium, phosphate, TNF-α and other interleukins, as well as novel factors such as FGF-23 and Klotho, on the local vitamin D metabolism in arteries.

**Results:** We have demonstrated the presence of VDR, 1α-OHase and 24-OHase in human kidney, artery and HAoSMCs. To our knowledge, we are the first to demonstrate the presence of 24-OHase in arteries. Our data suggested diminished expression of *VDR* mRNA and protein, with significantly decreased levels of 1α-OHase protein expression in CKD patients. Further crucial finding was the apparent increase of 24-OHase protein in CKD arteries suggested higher local vitamin D catabolism in CKD. In order to establish which factors regulate expression of 1α-OHase, 24-OHase and VDR in HAoSMCs, further studies were undertaken. Results confirmed that treatment with high calcium, phosphate, TNF-α, INF-γ, IL-6, IL-17A, FGF-23 or Klotho modulated VDR protein expression, which had further effect on expression of 1α-OHase and 24-OHase mRNA and protein.

**Conclusions:** The results suggest that altered mineral and inflammatory environment, characteristic to CKD may favour local $1,25(OH)_2D_3$ catabolism, potentially driving transdifferentiation of VSMCs, leading to VC. Vascular VDR activation, in particular through local metabolic activation, is crucial for vascular health, especially, under stress conditions. Local inducers and inhibitors of vascular vitamin D system have been determined, however further examination is required for potential application in future treatment of CKD related VC.

# Nomenclature

| *Abbreviation* | *Full name* |
|---|---|
| **25(OH)D** | 25-Hydroxyvitamin $D_2/D_3$ |
| **1,25(OH)$_2$D** | 1,25-Dihydroxyviatmin $D_2/D_3$ |
| **1α-OHase** | 25-Hydroxyvitamin D 1α-Hydroxylase, |
| **24-OHase** | Vitamin D 24-Hydroxylase |
| **ALP** | Alkaline Phosphatase |
| **BSA** | Bovine Serum Albumine |
| **bp** | Base pairs |
| **Ca** | Calcium |
| **CAMP** | Cathelicidin Antimicrobial Peptide |
| **$CaP_i$** | Calcium Phosphate Product |
| **cDNA** | Complementary DNA |
| **CKD** | Chronic Kidney Disease |
| ***CYP24A1*** | Vitamin D 24-hydroxylase gene product |
| ***CYP27B1*** | 25-hydroxyvitamin D 1α-hydroxylase gene product |
| **DBP** | Vitamin D Binding Protein |
| **DMEM/F-12** | Dulbeco Modified Eagle Medium F-12 |
| **EIA** | Enzyme Immunoassay |
| **eGFR** | estimated Glomerular Filtration Rate |
| **ESRD** | End Stage Renal Disease |
| **ECM** | Extracellular Matrix |
| **FCS** | Foetal Calf Serum |
| **FGF-23** | Fibroblast Growth Factor 23 |
| **FGFR** | Fibroblast Growth Factor Receptor |
| **HAP** | Hydroxyapatite |
| **HAT** | Histone Acetyltransferase |
| **HAoSMCs** | Human Aortic Smooth Muscle Cells |
| **HD** | Haemodialysis |
| **HKC-8** | Human Kidney Proximal Tubule Cell Line 8 |
| **IDBP** | Intracellular Vitamin D Binding Protein |
| **IGF-1** | Insulin-like Growth Factor 1 |
| **IHC** | Immunohistochemistry |
| **IL-6** | Interleukin 6 |
| **IL-17A** | Interleukin 17A |
| **INF-γ** | Interferon gamma |
| **KL1/2** | Klotho Domain 1 and 2 |
| **hrKlotho** | human recombinant Klotho |
| **mbKlotho** | membrane bound Klotho |

| | |
|---|---|
| **sKlotho** | soluble Klotho |
| **LVH** | Left Ventricular Hypertrophy |
| **MAARS** | Membrane Associated Rapid Response Steroid Binding |
| **mRNA** | messenger RNA |
| **MGP** | Matrix Gla Protein |
| **NiPi** | Sodium Dependent Phosphate Co-Transporter |
| **NO** | Nitric Oxide |
| **ON** | Osteonectin |
| **OPG** | Osteoprotegrin |
| **OPN** | Osteopontin |
| **$P/PO_4$** | Phosphate |
| **PBS** | Phosphate Buffered Saline |
| **PCR** | Polymerase Chain Reaction |
| **PTH** | Parathyroid Hormone |
| **PWV** | Pulse Wave Velocity |
| **PVDF** | Polyvinyl Difluoride |
| **RF** | Renal Failure |
| **RT** | Room Temperature |
| **RT - PCR** | Reverse Transcription – Polymerase Chain Reaction |
| **RXR** | Retinoid X Receptor |
| **SDS-PAGE** | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| **sHPT** | Secondary Hyperparathyroidism |
| **shRNA** | short harpin RNA |
| **TBS** | Tris Buffered Saline |
| **TBS-T** | Tris Buffered Saline with Tween |
| **TNF-α** | Tumor Necrosis Factor alpha |
| **TXD** | Transplant Donor |
| **TXR** | Transplant Recipient |
| **UVB** | Ultraviolet B |
| **VC** | Vascular Calcification |
| **VDR** | Vitamin D Receptor |
| **VDRA** | Vitamin D Receptor Activator/Agonist |
| **VDRE** | Vitamin D Response Element |
| **VSMCs** | Vascular Smooth Muscle Cells |

*Chronic Kidney Disease (CKD) is like every chronic condition, untreatable and unpreventable – in the UK alone, one CKD patient dies prematurely every 15 minutes - every other one due to the development of cardiovascular complications*[*].

Over 45,000 lives in the UK were lost between 2009 and 2010 due to CKD (Kerr 2012), which is equivalent to a disappearance of a whole population of Banbury or Lancaster. In USA the number of people diagnosed with CKD is growing and in 2010 it exceeded 20 million (CDC 2010) – the number of people inhabiting the largest city of the southern hemisphere, San Paulo. What is more, individuals with CKD are 16 to 40 times more likely to die than to reach ESRD. Over £1.45 billion were spent on CKD patient care between 2009 and 2010, where majority of the cost were dialysis and kidney transplantation. Over £8 million was spent in that time on vitamin D therapy (Kerr 2012).

This thesis investigates the role of vitamin D signalling in human artery in health and disease – all with a perspective of identifying factors for future application in treatment of CKD related cardiovascular complications.

[*] *Calculated based on data from (Kerr 2012).*

# Chapter 1

# General Introduction

## 1.1 Vitamin D and Artery in CKD

Vitamin D is essential for human health. The synthesis of the active vitamin D hormone $1,25(OH)_2D$, from the major circulating prohormone 25(OH)D is catalysed by the enzyme 25-hydroxyvitamin D 1α-hydroxylase (1α-OHase) and its catabolism is facilitated by vitamin D 24-hydroxylase (24-OHase). Generation of $1,25(OH)_2D$ circulating in the blood occurs in the kidneys and chronic damage and degeneration of kidney tissue, such as seen in chronic kidney disease (CKD) results in diminished blood levels of $1,25(OH)_2D$. This causes the abnormal calcium and phosphate homoeostasis, which is associated with CKD. $1,25(OH)_2D$ acts through binding to vitamin D receptors (VDR), which are present in almost every tissue of the human body, including arteries. In CKD activation of the VDR by $1,25(OH)_2D_3$ is attenuated, which can have deleterious effect on the physiology of arteries. The current understanding of the molecular mechanisms underlying uraemic arteriopathies is still quite basic. Vascular calcification (VC), one of the most important CKD pathology is currently untreatable and non-preventable, with the lack of ideal biomarkers.

This thesis aims to investigate the human vascular vitamin D system, its interaction with the endocrine system, changes in disease with focus on CKD with failure of the endocrine vitamin D system and lastly, regulation of the vascular vitamin D system.

## 1.2 Vitamin D

### 1.2.1 The History

The concept of vitamins, which were initially known as "vital amines" – organic micronutrients present in foods from natural sources and essential for health, emerged in early 19$^{th}$ century, following episodes of scurvy in sailors and endemic beriberi in Asian communities. Rickets, which is a condition induced by the vitamin D deficiency, was first described in the 15$^{th}$ century and later, more clearly by Whistler as "a condition in which the skeleton was poorly mineralised and deformed", however, the cause was still a mystery (Whistler 1645). The end of 19$^{th}$ century witnessed a significant emergence of rickets in the low-sun light countries, especially in England, where the condition was known as an "English disease". This epidemic of rickets struck during industrial revolution, when the rate of urbanisation increased, ultimately leading to atmosphere pollution further reducing peoples' exposure to sun light (Hess 1929). Cod liver oil has been suggested to have a beneficial effect in the treatment of English disease, although, initially not much attention was paid (DeLuca H. 2005). McCollum was an American scientist, who by studying fat soluble properties of vitamin A in cod liver oil, has shown that an aeration and heat inactivation of this nutrient has not compromised the ability of cold liver oil to cure rickets, suggesting that there was another essential nutrient. This was how McCollum discovered vitamin D (McCollum 1922). Further experiments on

dogs, cows, chickens and goats have led scientists to rightly associating the sun exposure with calcium balance in animal body. The irradiation of plant sterols and eventually human skin with ultraviolet light has led first to isolation of ergocalciferol - vitamin $D_2$ and six years later, 7-dehydrogestrel – the human precursor of vitamin $D_3$ by Askew *et al.* (Askew 1931, Windaus 1937).

Only four decades ago, 25(OH)D was thought to be the active form of vitamin D (Blunt 1968). The scientific curiosity, however, has turned out to have phenomenal consequences, when few years after Blunt's discovery, Fraser and Kodicek disproved his hypothesis, to confirm that $1,25(OH)_2D$, was the peak metabolite in the analysed chick kidney homogenate, pointing out the kidney as the site of vitamin D final activation step (Fraser DR; Kodicek, E. 1970).

It is now known that $1,25(OH)_2D$ is not a true vitamin (because healthy individuals with adequate exposure to sunlight do not require dietary supplementation), it is a steroid hormone, which when acting via the endocrine way is responsible mainly for mineral homeostasis and bone growth.

In recent years it was suggested that $1,25(OH)_2D$ can be synthesised in various other tissues of the human body, such as macrophages, keratinocytes, pancreatic β-cells, neurons, parathyroid, bone and many more, suggesting other roles for this astonishing compound, such as acting on proliferation, differentiation and immunity (Adams and Gacad 1985, Bland *et al.* 2001, Bland *et al.* 2004, Equils *et al.* 2006, Hewison *et al.* 1994, Jones *et al.* 1998, Liu Philip 2006, Stoffels *et al.* 2006, Zehnder *et al.* 2001, Zehnder *et al.* 2002a). The topic remains controversial, as some strongly argue against the existence of extra-renal $1,25(OH)_2D$ synthesis (Vanhooke *et al.* 2006).

## 1.3 The Vitamin D Hormonal System

### 1.3.1 Metabolic Origin, Activation and Catabolism

There are two different isoforms of vitamin D - $D_2$ and $D_3$ and both are thought to be biologically inert. Provitamin D is either produced in the skin (cholecalciferol - $D_3$), under the exposure to ultraviolet B (UVB) light at 290-315 nm wavelengths, or ingested by consuming oily fish, some plants (especially from *Solanaceae* family: potatoes, tomatoes, aubergines, chilli peppers and tobacco), (ergocalciferol – $D_2$). UVB mediates a photolytic, non-enzymatic reaction that converts endogenous 7-dehydrocholesterol into a previtamin $D_3$. Following that, the precursor of the active hormone undergoes a non-enzymatic thermal isomerisation conversion to vitamin $D_3$, which via circulation is transported to the liver (since vitamin $D_2$ is ingested, it is absorbed through the gut and transported with blood to the liver, together with vitamin $D_3$. In the liver, vitamin $D_2$ and $D_3$ further undergo hydroxylation to produce 25(OH)D (calcidiol), as depicted below (*Figure 1.1*), which is the main circulating pro-hormone. It is the most abundant form of vitamin D and exists in circulation bound to vitamin D binding protein (DBP), which stabilises it. In fact 25(OH)D is a mixture of 25(OH) $D_2$ and 25(OH)$D_3$, however for the simplicity, throughout this thesis calcidiol it will be referred to as 25(OH)D. Levels of 25(OH)D < 5 ng/ml or 12 nM are indicative of severe vitamin D deficiency, 5-15 ng/ml or 12-37 nM indicate mild deficiency and those of 16-30 ng/ml or 40-75 nM – 25(OH)D insufficiency. The second hydroxylation and bio-activation stage occurs primarily in the proximal tubule cells in the kidneys, where the renal 1α-OHase, produces the circulating fraction of the active form of vitamin D, the sterol hormone - 1,25$(OH)_2$D (calcitriol). 24-OHase, which is present in virtually all vitamin D target tissues

catabolises both 25(OH)D (to 24,25(OH)$_2$D) and 1,25(OH)$_2$D (to 1,24,25(OH)$_3$D, as represented by *Figure 1.2*.

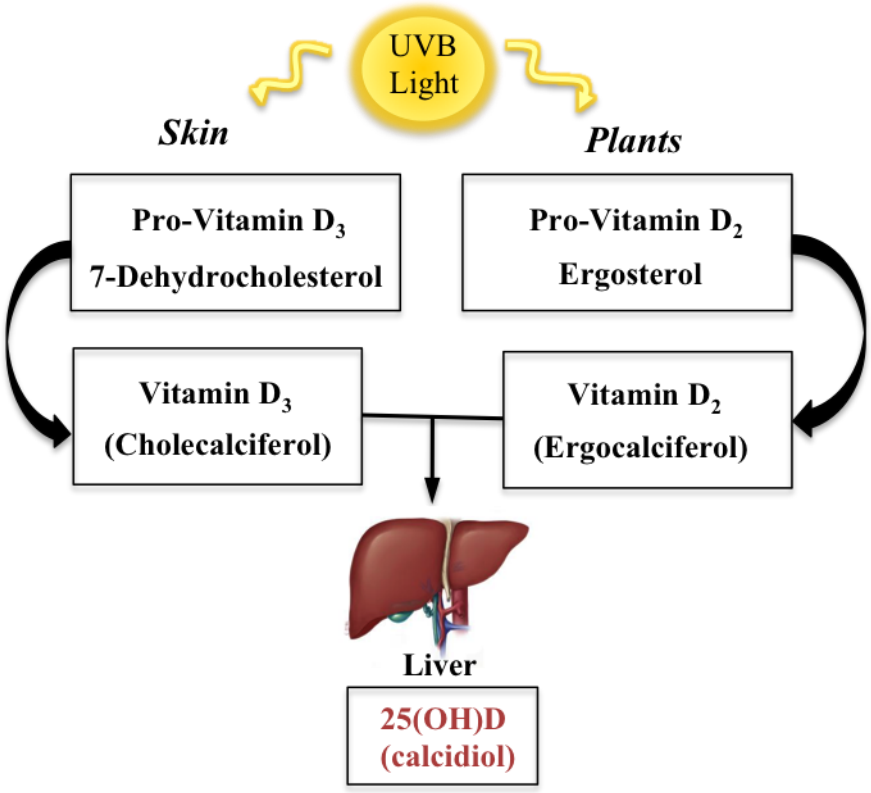


***Figure 1.1: Initial stages of vitamin $D_2$ and $D_3$ synthesis from natural precursors, in humans and in plants.***

### 1.3.2 Receptors and Other Proteins Involved in Vitamin D Signalling

The majority of 1,25(OH)$_2$D actions are mediated by its binding to the high-affinity nuclear receptor, the vitamin D receptor (VDR). This complex then forms a heterodimer with retinoid X receptor (RXR), which binds to vitamin D response elements (VDREs) in the promoter of 1,25(OH)$_2$D-responsive genes. The rate of transcription by the VDR is regulated by the recruitment of nuclear co-regulator

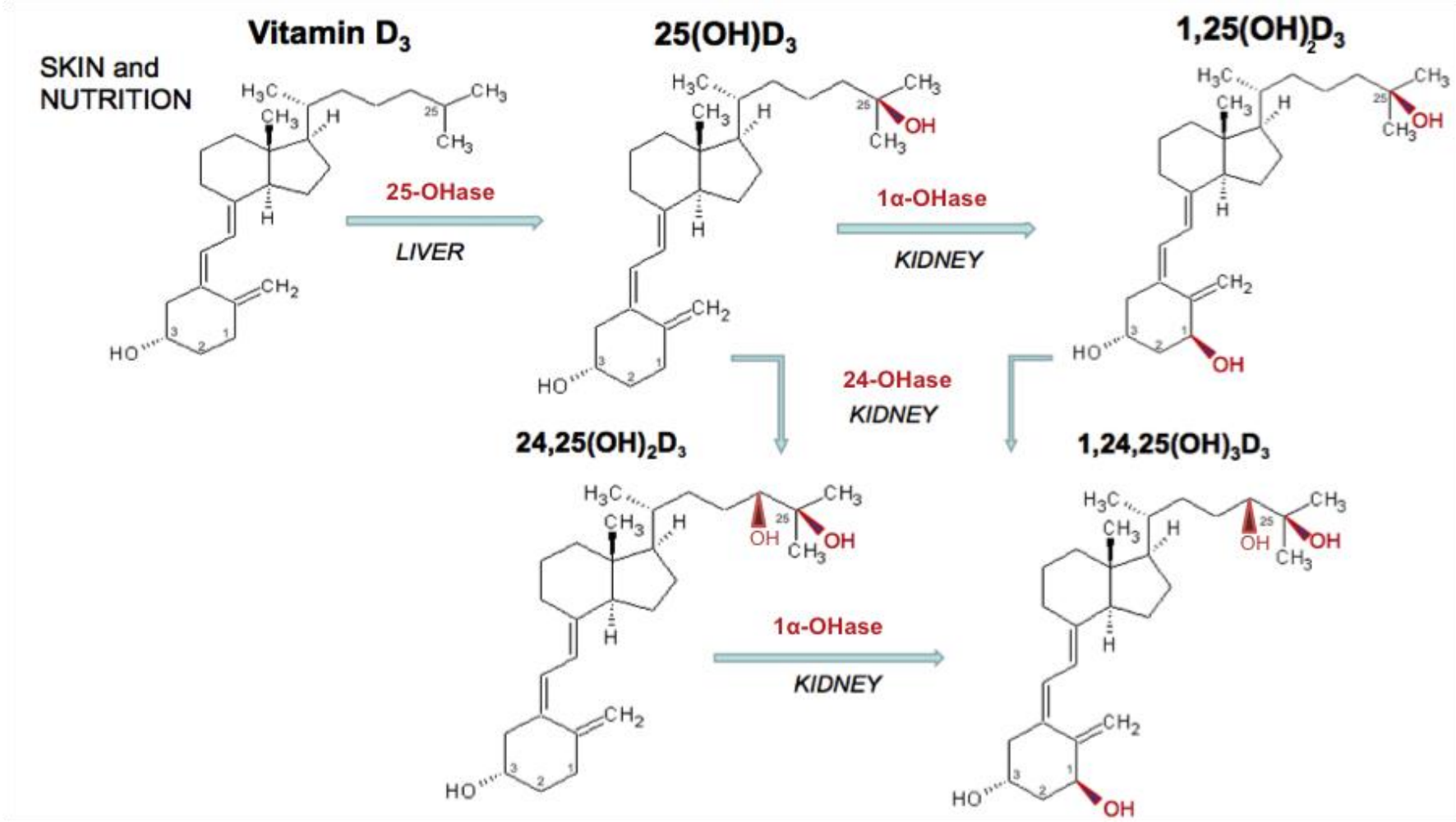


***Figure 1.2: Vitamin D metabolism.*** *The image is a schematic representation of the multiple step process required for the production of fully active 1,25(OH)$_2D_3$ in the kidneys. Vitamin D 25-Hydroxylase (25-OHase), 25-Hydroxyvitamin D 1α-Hydroxylase (1α-OHase), vitamin D 24-Hydroxylase (24-OHase).*

proteins into the transcription pre-initiation complex, as pictured below *(Figure 1.3).* VDR localisation is not restricted to nucleus, as presence of functional VDR was shown in cavolae-enriched plasma membrane, mitochondria, cytosol and perinuclear space (Gonzalez Pardo *et al.* 2008, Huhtakangas *et al.* 2004, Nemere *et al.* 1994). Furthermore, t-tubules of rat cardiac myocytes have also been shown to colocalise with VDR. Portion of these t-tubule bound VDR receptors upon exposure to $1,25(OH)_2D_3$ is translocated to nucleus. The remaining VDR is thought to be positioned ideally to exert an immediate effect on signal transduction mediators and ion channels, ultimately playing role in heart structure and function (Tishkoff *et al.* 2008).

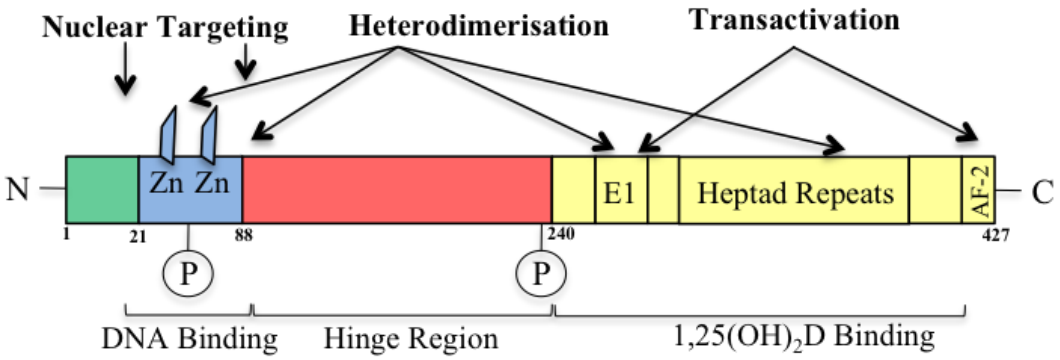


***Figure 1.3: Schematic representation of the VDR mRNA transcript.***

### *1.3.2.1 Vitamin D Binding Protein*

Since Vitamin D and its hydroxylated metabolites are lipophilic compounds, in order to be efficiently transported in plasma they must be bound to plasma proteins. Plasma proteins, such as DBP, albumin and some lipoproteins are present in excess compared to vitamin D metabolites and play an important role in extending their circulation half-life. The second most abundant serum glycoprotein is DBP - it binds to 25(OH)D and $24,25(OH)_2D$ with highest affinity, and not as strongly to

$1,25(OH)_2D$ (Cooke and Haddad 1989). The ability of DBP to bind to $1,25(OH)_2D$ is a mechanism that could have been developed to help to prevent intoxication (Bouillon *et al.* 1981).

#### *1.3.2.2 Proteins Involved in Crossing the Membrane*

25(OH)D-DBP complex is sequestered by cubilin on the cell surface to further bind to the endocytic megalin receptor and the receptor-associated protein (RAP), which facilitates its uptake (Birn *et al.* 2000, Nykjaer *et al.* 2001). Megalin has also the ability to bind the VDR co-activator SKI-interacting protein (Skip), which highlights another way of controlling the vitamin D signalling (Petersen *et al.* 2003).

Megalin-mediated uptake may not be the only mechanism facilitating entry of vitamin D metabolites inside the cell. Interestingly, stimulation of various cell types with $1,25(OH)_2D$ induced fast (too fast to be mediated by the nuclear VDR action) cellular responses, so called 'non-genomic' responses, such as increase in inositol triphosphate, diacylglycerol, intracellular calcium and intestinal phosphate fluxes (de Boland *et al.* 1994, Karsenty *et al.* 1985, Norman A. 1997, Slater *et al.* 1995, Wali *et al.* 1990). PKC, membrane bound VDR and more recently, membrane caveolae and membrane-associated rapid response steroid-binding (MARRS) have been suggested as possible membrane receptors (Nemere and Farach-Carson 1998, Nemere *et al.* 1998, Slater *et al.* 1995). Binding of $1,25(OH)_2D$ may trigger activation of single or multiple second messenger systems, including phospholipase C (PLC), G-protein coupled receptors and protein kinase C (PKC) or phosphatidylinositol-3-kinase (PI3K), which may result in opening of the voltage-gated calcium channels or chloride channels, further facilitating the cross-talk with the nucleus modulating the gene expression.

#### *1.3.2.3 Intracellular Vitamin D Binding Protein (IDBP)*

The intracellular part of the megalin receptor has been shown to interact with intracellular vitamin D binding proteins 1 and 2 (IDBP1 and IDBP2), which are homologous to the heat-shock proteins. Upon 25(OH)D-DBP complex entry into the cell, DBP is degraded by legumain, a naturally occurring cysteine endopeptidase. This allows the release of 25(OH)D, which remains bound to IDBP, responsible for targeting the metabolite to the inner mitochondrial membrane.

#### *1.3.2.4 The Vitamin D Receptor*

VDR belongs to the steroid-thyroid superfamily of nuclear receptors and it has been cloned first in chicken (McDonnell *et al.* 1987). Few years later the presence of VDR was shown in almost every human tissue, except red blood cells, highly differentiated brain cortex and mature striated muscle (Krill *et al.* 2001, Menezes *et al.* 2008, Perez-Fernandez *et al.* 1997, Segaert *et al.* 1998, Wu-Wong *et al.* 2007b, Zanello *et al.* 1997). VDR is homologous to other nuclear receptors, such as retinoid receptors (RXR) and constitutive androstane receptors (CAR), which bind bile acid metabolites and are able to activate CYP enzymes involved in drug detoxification. Interestingly, the presence of VDR has also been detected in jawless primitive fish (*Petromyzon marinus*) suggesting that vitamin D system emerged even before the development of calcified structures, perhaps serving as a detoxifying mechanism (Whitfield *et al.* 2003). VDR acts via dimerising with any of the three RXR isomers. Both VDR and RXR are inactive when not bound to their ligands, 1,25$(OH)_2$D and 9-cis-retinoic acid, respectively. There are at least three different VDR splice variants- two of them produce 427 amino acid long protein (difference is in 5' untranslated region) and one a 477 amino acid long protein, due to 50 amino acid N-

terminal extension). The longer protein structural variant is called VDR-B1 and makes up about 30% of total VDR found in kidney. *In vitro* studies using different cell lines derived from kidney (COS1 and HEK293) breast (HS578T and MCF7) and intestine tissue (Caco-2, LS174T and HCT15) have shown that there are differences in the transactivation capacity of the 24-hydroxylase promoter in each cell-line (Esteban *et al.* 2005). These differences seemed to be attributed to the to the fact that A/B domains possess promoter- and cell context-dependent activity proposing that the VDR N-termini may interact with cell-specific co-factors (McInerney *et al.* 1998, Tzukerman *et al.* 1994). VDR consists of two main domains – DNA binding domain (DBD) and ligand binding domain (LBD), linked by the hinge region, as depicted on the *Figure 1.3*. The DBD comprises two zinc fingers, which are essential for interaction of VDR with DNA regulatory elements and nuclear translocation of VDR from cytosol. Mutations in this area can be one of the causes of VDR resistance in humans and mice, they also inevitably lead to alopecia (Haussler 1986, Hsieh *et al.* 1998, Malloy and Feldman 2003). The hinge region is longer than in other nuclear receptors ensuring sufficient flexibility for DNA-DBD interaction as well as the LBD-co-activators interaction. The LBD is formed by twelve α-helixes and three β-sheets, folded to form a large pocket allowing accommodation of a long $1,25(OH)_2D$ and anaologues' side chain. Mutations in the LBD impair or abolish binding of the ligand (Bouillon *et al.* 2008). Activation factor-2 (AF-2) domain provides a platform for binding of co-activator proteins. Multiple polymorphisms have been identified in humans along the VDR gene, which have later been associated with cancer, diabetes, arthritis and other disorders (Arai *et al.* 1997, Nejentsev *et al.* 2004a, Nejentsev *et al.* 2004b, Uitterlinden *et al.* 2004).

#### *1.3.2.5 Vitamin D Response Element (VDRE) and Co-Activator and Co-Repressor Proteins*

In response to $1,25(OH)_2D$, VDR is phosphorylated, which causes structural reconfiguration and leads to the release of co-repressors. Studies using mice revealed for the first time that VDR dimerises with RXR, which allows the complex to bind to two hexameric VDRE binding sites (Schrader *et al.* 1997). Genes regulated by VDR can have multiple VDREs present in their promoter (Meyer *et al.* 2006).

Nuclear co-regulator molecules play an important role in positive and negative modulation of VDR. Two regions are of particular importance – residue 246 of the RXR heterodimerisation domain (E1) and the AF-2 domain. Nuclear co-activators, such as SRC-1 p160 and CBP/p300, which poses the histone acetyl transferase (HAT) DNA unfolding activity, act together with VDR to increase the effect $1,25(OH)_2D$ has on gene transactivation. Further, a complex of approximately 15 transcriptional co-activators (DRIP-TRAP) is recruited and binds directly to VDR via DRIP205 forming a bridge with the basal transcription machinery creating a transcription pre-initiation complex to stimulate VDR-induced gene expression *(Figure 1.4)* (Dusso *et al.* 2005, Jurutka *et al.* 2001, Rachez and Freedman 2000).

VDR can also act to repress expression of certain genes, for instance the one coding for PTH. In such situation, binding of the VDR-RXR complex to a negative VDRE recruits co-repressors, which de-acetylates histones preventing chromatin exposure, ultimately blocking binding of the transcriptional machinery. Interestingly, other set of VDR modulators, different to co-activators and co-repressors has been described. NCoA62/Skip has a double role – it can promote activation or repression, depending on the expression of other co-regulator molecules (Jurutka *et al.* 2001, Leong *et al.* 2004, Rachez and Freedman 2000).

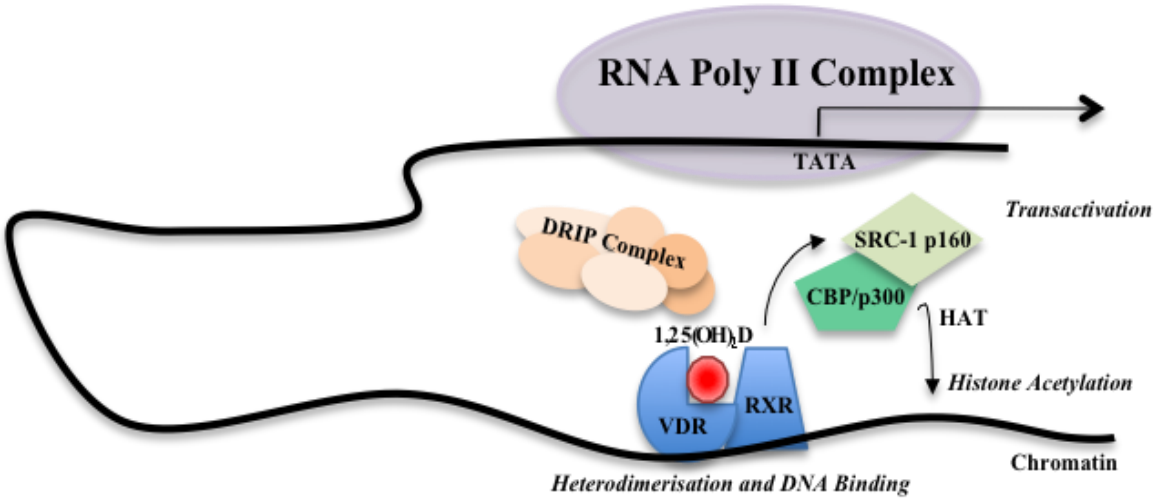


***Figure 1.4: Schematic representation of steps necessary for gene transcription from the Vitamin D Response Element (VDRE).*** *Histone Acetyl Transferase (HAT), Vitamin D Receptor Interacting Protein (DRIP), Vitamin D Receptor (VDR), Retinoid X receptor (RXR), polymerase (Poly), CREB binding protein/EA1 binding protein (CBP/p300), Steroid Receptor Co-activator 1/nuclear co-activator (SRC-1/ p160).*

### 1.3.3 Enzymes Involved in Vitamin D Metabolism -The Cytochrome P450 Enzyme System

Mixed function oxidases are the enzymes responsible for the stereo-specific hydroxylation of steroids. They catalyse reduction of one atom of molecular oxygen to water and one atom of the hydroxyl group to be attached to the steroid or sterol hormone. The Cytochrome P450 enzymes are involved in the synthesis of essential cholesterol derived hormones, such as estrogens, androgens, mineralocorticoids, glucocorticoids and vitamin D. There are two types of cytochrome P450 enzymes, type 1 includes microsomal enzymes, such as 17α-OHase, 21-OHase, hepatic enzymes and aromatases. Within the microsomal endoplasmatic reticulum flavoprotein serves as an electron donor, passing the electrons to the enzymes. Type

2 P450 enzymes are localised in inner mitochondrial membrane and comprise major vitamin D regulating enzymes such as 25-OHase, 1α-OHase and 24-OHase.

The electrons required for the oxygen reduction are derived from nicotinamide adenine dinucleotide phosphate (NADPH, reduced form), which is generated intramitochondrially during the tricarboxylic acid metabolism. They are then transferred to NADPH-ferredoxin reductase and ferredoxin (non-haeme iron-sulphur protein), which shuttles between mitochondrial matrix and the cytochrome P450 (specific for each hydroxylase) (Solish *et al.* 1988).

#### *1.3.3.1 25-Hydroxylation: The Vitamin D 25-Hydroxylase (25-OHase)*

25-OHase, an enzyme found in the hepatic mitochondria, requires two mitochondrial protein cofactors: ferredoxin and ferredoxin reductase in order to function (Hanukoglu and Gutfinger 1989). Interestingly, intestine, keratinocytes, kidney, macrophages have been confirmed as an extra-hepatic site of 25-hydroxylation, after original studies by Olson, in which hepatectomy was shown to only reduce, but not eliminate the plasma 25(OH)D (Bjorkhem *et al.* 1994, Lehmann *et al.* 1999, Olson *et al.* 1976). 25-OHase is a multifunctional enzyme, with the same enzyme expressed in various tissues. Its activity is not restricted to vitamin D metabolism, as it can also hydroxylate other intermediates of cholesterol synthesis, (Okuda *et al.* 1995). 25-OHase is involved in the cholesterol clearance from the extra-hepatic tissues (Cali *et al.* 1991, Leitersdorf *et al.* 1994). Out of vitamin D hydroxylases, it is the most stable one, with the half-life of about 24 hours, ensuring a slow response to modulation (Stravitz *et al.* 1996). 25-OHase is suppressed by PTH, levels of which are increased

in CKD, ultimately resulting in diminished hepatic synthesis of 25(OH)D in uraemia (Michaud *et al.* 2010).

#### *1.3.3.2 1α-Hydroxylation: The 25-Hydroxyvitamin D 1α-Hydroxylase (1α-OHase)*

Studies on VDR-knockout mice overexpressing 1α-OHase allowed cloning of *CYP27B1* cDNA (Takeyama *et al.* 1997). Evidence from further genetics studies on humans shows that renal and extra-renal 1α-OHase arise from the same gene product giving rise to proteins with 501 to 508 amino acids. The gene *CYP27B1* has been mapped to chromosome 12 and is split into nine exons (I-IX), where seven are of a similar size 170–200 bp encoding 57–67 amino acids each and two smaller ones, encoding 26 and 37 amino acids. The $1,25(OH)_2D$ binding region is encoded by exon VI and the haeme-binding region, positioned around the cysteine residue that is present in all mitochondrial P450s is encoded by exon VIII. The intron size ranges from approximately 80 to 660 bp, overall making the *CYP27B1* gene small in size compared with other mitochondrial cytochrome P450s. The intron/exon organisation is highly conserved across the genes coding for these enzymes (Diaz L. *et al.* 2000b, Fu *et al.* 1997, Monkawa *et al.* 1997, St-Arnaud *et al.* 1997). 1α-OHase protein is highly conserved between species and knockout in mice results in development of hypertension, cardiac hypertrophy, impaired cardiac function, together with an up-regulation of the renin-angiotensin system in both renal and cardiac tissues (Zhou *et al.* 2008).

1α-OHase is a mixed function oxidase, which reduces one atom of molecular oxygen to water and allows 1α-hydroxyl group to be stereospecifically attached to the A ring of the sterol – 25(OH)D, which produces $1,25(OH)_2D$ (Midgett *et al.* 1973). The use

of a highly specific assay based on isotope-dilution mass spectrometry showed that human mitochondrial 1α-OHase has a maximal hydroxylation velocity ($V_{max}$) of 1.7 ± 0.2 μM/min/mg protein and the apparent Michaelis constant ($K_m$) of 14 μM *(where $K_m$ is a concentration of substrate required to reach half of the maximal velocity*) (Hagenfeldt and Berlin 1992). These findings differ significantly from previous reports of Fu and co-workers, where the $K_m$ for 1α-OHase was found to be 0.27 μM (Fu *et al.* 1997). Other reports from studies using a cloned human 1α-OHase cDNA into E.coli JM109 showed values similar to those of Fu – 24,25$(OH)_2$D had a lower $K_m$ value (1.1 μM) and higher $V_{max}$, compared to 24(OH)D, Km (2.7 μM) and lower $V_{max}$, which indicated that 24,25$(OH)_2$D would be a better substrate for 1α-OHase than 25(OH)D (Sawada *et al.* 1999).

Studies on rabbits, chicks and humans have shown strong 1α-OHase activity in the inner mitochondrial membrane of proximal tubule cells of the kidney (Akiba *et al.* 1980, Brunette *et al.* 1978, Kawashima *et al.* 1981, Zehnder *et al.* 1999).

The important regulators of renal 1α-OHase activity are 1,25$(OH)_2$D, PTH, FGF-23, calcitonin, dietary calcium and phosphate, and also other steroidal hormones such as estrogen. Studies on kidneys of intact animals and human proximal tubule cells (HKC-8) cells have shown that 1,25$(OH)_2D_3$, calcium and phosphate inhibit *CYP27B1* mRNA and stimulate increase in *CYP24A1* gene transcription (Bland *et al.* 1999, Murayama *et al.* 1999, Rosenthal *et al.* 1980, Zierold *et al.* 2000). The exact mechanism may involve regulation of the promoter activity leading to a decrease in mRNA or an indirect transcriptional process or post-transcriptional/post-translational modifications. Further, experiments on parathyroidectomised animals have demonstrated diminished 1α-OHase activity and consequently PTH treatment has

been shown to induce *CYP27B1* levels above basal (Armbrecht *et al.* 2003, Booth *et al.* 1985, Walker *et al.* 1990). Increase in PTH induces cAMP and the probol esther protein kinase C (PKC) in renal cells, which results in induction of *CYP27B1* expression (Armbrecht *et al.* 2003, Dunlay and Hruska 1990). cAMP and forskolin, (which is a labdane diterpene, a chemical produced by a plant *Coleus forskohlii* previously shown to strongly induce adenylate cyclase) both independently exert the same action on 1α-OHase (Fukase *et al.* 1982, Henry H. L. 1985). PKC activator – TPA, also mimics the action of PKC in rat model, but not in the chick, where 1α-OHase and 24-OHase inhibition has been observed (Ro *et al.* 1992, Tang C. *et al.* 1993). The inhibition of 1α-OHase expression by 1,25$(OH)_2$D and TPA has additive effect, indicating that both regulators act via distinct pathways. Previous studies *in vitro* and using normocalcaemic rat models have shown contrary data as to the effect of calcitonin on renal 1α-OHase. In human, calcitonin appeared to upregulate 1α-OHase promoter, similarly in rats, 1α-OHase expression was increased, but VDR levels dropped (Shinki *et al.* 1999, Yoshida N. *et al.* 1999). Opposite results or no change were reported in a number of *in vitro* studies, suggesting that the regulation of calcium homeostasis by calcitonin may require actions of other elements present in the circulation (Henry HL. *et al.* 1982 , Larkins *et al.* 1974, Rasmussen *et al.* 1972).

#### *1.3.3.3 24-Hydroxylation: The 1,25-Dihydroxyvitamin D 24-Hydroxylase (24-OHase)*

The human 24-OHase gene *CYP24A1* has been mapped to chromosome 20 at q13.2-q13.3 (Labuda *et al.* 1993). It is about 15 kb in length and so far, no known diseases have been linked to this gene. Just like 25-OHase and 1α-OHase - 24-OHase is also a mixed-function monooxigenase. It metabolises 25(OH)D to 24,25$(OH)_2$D

(significant serum levels of 2-5 ng/ml; $1/5^{th}$ to $1/10^{th}$ of the 25(OH)D concentration) and 1,25$(OH)_2$D to 1,24,25$(OH)_3$D (concentration thought to be insignificant). The catalysis involved binding oxygen and directing the regiospecific and stereospecific hydroxylation and oxidation of secosteroid substrate molecule utilising electrons obtained from NADPH (Ohyama and Okuda 1991). Previous enzyme kinetics studies have shown that $K_m$ values for 1,25$(OH)_2$D are 1/4 to 1/30 that of 25(OH)D, indicating that 24-OHase has higher preference for 1,25$(OH)_2$D. Other studies have shown directly opposite results, which may be explained by the sequestrial-multicatalytic properties of 24-OHase together with the effect of the electron transfer and dissociation of intermediate reactants from the enzyme (Taniguchi *et al.* 2001).

The 24-OHase is expressed in a variety of VDR-expressing tissues, including kidney, intestine, bone, macrophages, and keratinocytes as well as in colon, prostate and mammary cancers (Akeno *et al.* 1997, Fischer *et al.* 2009, Lechner *et al.* 2007, Ornoy *et al.* 1978, Overbergh *et al.* 2000, Xie *et al.* 2002, Yang *et al.* 1999). It localises to inner mitochondrial membrane, just like 1α-OHase (Ohyama and Okuda 1991).

Due to its broad tissue distribution, the expression of *CYP24A1* is influenced by a vast range of regulatory agents. In kidney and osteoblasts 1,25$(OH)_2$D is the strongest inducer of *CYP24A1 (Figure 1.5).* Studies of *CYP24A1* rat promoter revealed 2 tandem-arranged VDREs constitute the *CYP24A1* promoter. *VDRE-1* and

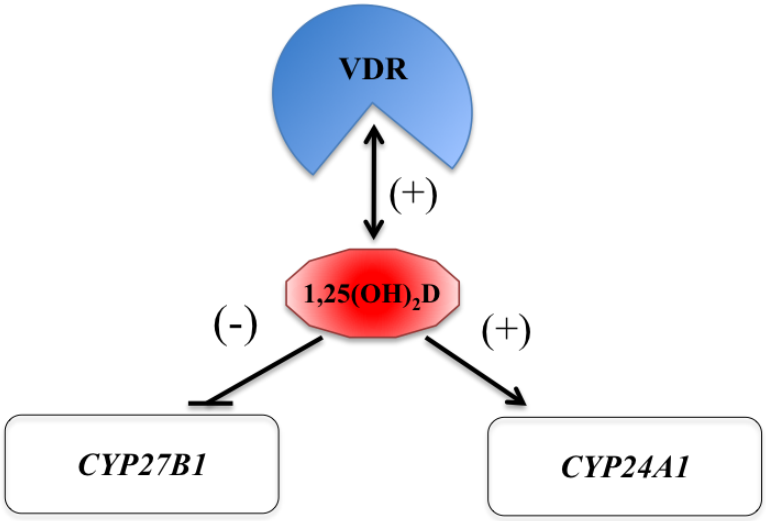


***Figure 1.5: The endocrine effect of 1,25(OH)$_2$D on Vitamin D Receptor (VDR), 1α-Hydroxylase gene product (CYP27B1) and 24-Hydroxylase gene product (CYP24A1).*** *(+) induction, (-) inhibition.*

*VDRE-2* were found on the non-coding strand, about 100 bp apart. Low expression in the absence of 1,25(OH)$_2$D is maintained by a repressor complex bound to unliganded VDR/RXR. When 1,25(OH)$_2$D is abundant, it binds to VDR, it is thought that the repressor complex dissociates and is replaced by the co-activator complex with the histone acetyltransferase (HAT) activity, which role is to unwind the chromatin, to ultimately be replaced by the mediator complex (Sutton and MacDonald 2003). This last step would stimulate interaction with RNA polymerase II and ultimately induce *CYP24A1* gene transcription (Omdahl J; May, BK; 2005, Vidal *et al.* 2002). Recent studies demonstrated a micro RNA (miRNA) miR-125b recognition element (MRE125b) in the 3'-untranslated region of human *CYP24A1*. Using normal and cancerous breast tissue it has been confirmed that *CYP24A1* mRNA can also be affected by dysregulated miRNA (Komagata *et al.* 2009).

#### *1.3.3.4 Other Metabolic Pathways*

There are numerous other complex vitamin D metabolic pathways, but the physiological significance of some is yet to be clarified. Over 30 vitamin D metabolites have been identified, but only some of them have been shown to be biologically active. 24-OHase can use both 25(OH)D and 1,25$(OH)_2$D as substrates and for both can catalyse reactions at carbon 23 (C-23) as well as C-24. 24-OHase can produce at least 18 forms of metabolites in human (Corvol *et al.* 1978, Henry H. L. and Norman 1978, Ornoy *et al.* 1978, Sakaki *et al.* 2000, Shima *et al.* 1990). The enzyme was initially shown to be able to induce three successive oxidations – two at carbon C-24 and one at carbon C-23, producing an intermediate that is further cleaved to calcitroic acid (from 1,25$(OH)_2$D substrate), a metabolite that is thought to have very low biological activity (Beckman *et al.* 1996). More recent studies confirmed that C-23 hydroxylation pathway of 25(OH)D in fact consists of four-step monooxygenation. Below are pictured the two parallel pathways of 25(OH)D and 1,25$(OH)_2$D catabolism by 24-OHase *(Figure 1.6)* All four reactions occurring on the side chain are the same and are catalyzed by a single active site on 24-OHase (Henry H. L. 2011, Sakaki *et al.* 2000).

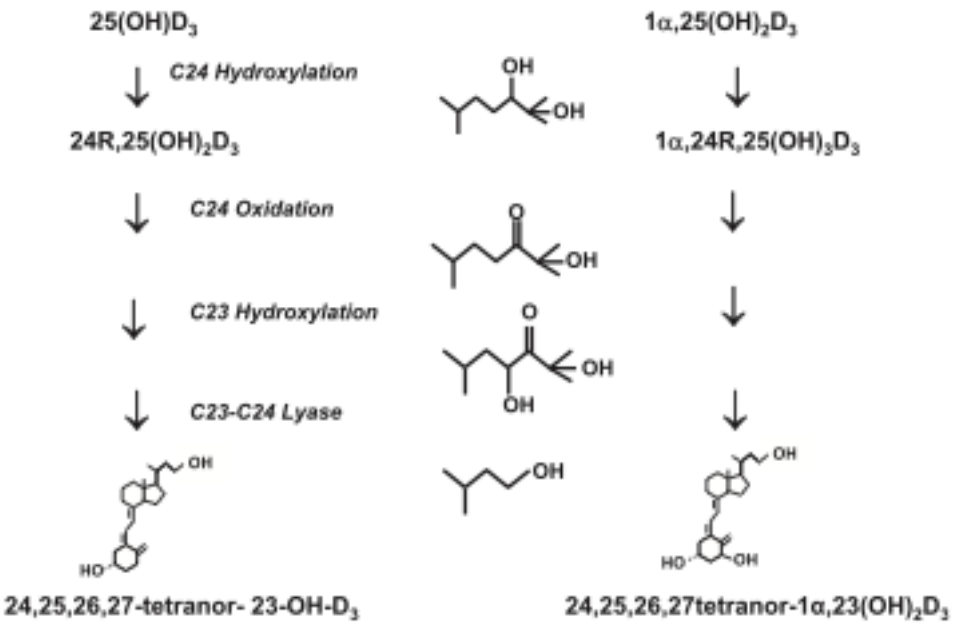


***Figure 1.6: The reactions facilitated by the 25(OH)D-/1,25(OH)$_2$D-24 Hydroxylase (24-OHase)***. *24-OHase catalyses catabolism of both 25(OH)D and 1,25(OH)$_2$D, two parallel pathways are pictured. Reproduced with permission from (Henry H. L. 2011)*

Recent evidence indicates that a single specific mutation in *CYP24A1* converts human 25(OH)D 24-OHase into 23-OHase, generating 1,25(OH)$_2$D-26,23-lactone (Prosser *et al.* 2007). It has been suggested that some 24-OHase metabolites can be potent VDR antagonist (Ishizuka *et al.* 2005, Toell *et al.* 2001, St Arnaud R. 2010).

### 1.3.4 Knockout in Animals

#### *1.3.4.1 VDR Knockout*

Critical analysis of mice with engineered deletion of *VDR* gene, allowed re-evaluation of the specific mode of action and the spectrum of activities of 1,25(OH)$_2$D and its metabolites. The VDR expression is ubiquitous in both mice and human cells, with almost all cells responding to 1,25(OH)$_2$D. Most astonishing is the

fact that approximately 3% of the mouse and human genome is regulated by the vitamin D hormonal system.

VDR-knockout mice exhibited a normal phenotype at birth. With age their growth retards and mice develop hypocalcaemia, hypophosphataemia, secondary hyperparathyroidism, severe rickets and osteomalacia (Li *et al.* 1997, Van Cromphaut *et al.* 2001, Yoshizawa *et al.* 1997). Despite the lack of functional VDR, serum levels of $1,25(OH)_2D$ levels were increased (increased 1α-OHase activity and decreased 24-OHase activity) due to secondary hyperparathyroidism (Amling *et al.* 1999, Yoshizawa *et al.* 1997).

Further, *VDR* (or *CYP27B1*) knockout in mice that were subsequently maintained on a rescue diet resulted in hyperproliferation of distal colon cells, as measured by increased levels of cyclin D1. Furthermore, it also lead to the direct DNA damage, as evaluates by increased levels of 8-hydroxy-2-deoxyguanosine (Kallay *et al.* 2002, Kallay *et al.* 2001). Other studies using *VDR* or CYP27B1-knockout mice demonstrated that skin of these animals was significantly more prone to tumorigenesis, following oral administration of carcinogen. The skin of *VDR*-knockout mice not exposed to carcinogen, aged faster, become wrinkled and cystitic (Zinser *et al.* 2002). *VDR* ablation has been positively correlated tumour growth in epithelia of the mammary gland (Welsh 2004). Tumor development in ovary, uterus, lung, or liver was not different between *VDR* wild type and *VDR*-knockout mice. Interestingly, lung metastatic cancer growth was hugely reduced in VDR-knockout mice, which may suggest that optimal VDR signalling may contribute to suppression of carcinogenesis (Nakagawa *et al.* 2004).

Importantly, there is a clear relationship between the presence of a functioning VDR and healthy vasculature, as *VDR* knockout in mice resulted in the development of

hypertension, elevated trombogenicity and cardiac hypertrophy (Bouillon *et al.* 2008).

#### *1.3.4.2 CYP27B1 Knockout*

Mice with engineered deletion of *CYP27B1* gene phenotypically resembled those with *VDR* gene knockout. Both displayed severe hypocalcaemia and hypophosphotaemia. Conversely to *VDR*-knockout mice, in mice with deficient *CYP27B1* the serum $1,25(OH)_2D$ levels were undetectable and 25(OH)D levels were elevated. Also, $24,25(OH)_2D$ levels were decreased compared to VDR null mice (Dardenne *et al.* 2003, Panda *et al.* 2001, Rowling *et al.* 2007, Van Cromphaut *et al.* 2001). Similar observations have been made in patients with pseudovitamin D-deficiency rickets (Balsan *et al.* 1986, Kitanaka *et al.* 1998).

The protective effect of $1,25(OH)_2D$ on vasculature was demonstrated, during studies of the Wistar rat aorta organ culture. Data revealed that the treatment with $1,25(OH)_2D$ had an effect on myosin production, overall enhancing contractility (Ishibashi and Bukoski 1997). More recent evidence from studies on *CYP27B1*-knockout mice suggests that administration of $1,25(OH)_2D_3$ not only normalised serum calcium and phosphorus levels, but also normalised blood pressure, cardiac structure and function and the renin-angiotensin system (Zhou *et al.* 2008).

#### *1.3.4.3 CYP24A1 Knockout*

*CYP24A1* knockout animal models include mice, rats, pigs and chicks (Akeno *et al.* 1997, Chen K. S. *et al.* 1993, Jehan *et al.* 1998, Ohyama *et al.* 1991, Zierold 2001). There are no known human diseases that can be attributed to *CYP24A1* gene deficiency. *CYP24A1*-knockout mice suffer from severe hypercalcaemia and

nephrocalcinosis, due to excess of 1,25$(OH)_2$D that simply cannot be catabolised. They also exhibit excessive undermineralised bone matrix and hardly ever survive past neonatal period (Hock *et al.* 1986). This was readdressed in a recent study in mice, which demonstrated that maintaining normocalcaemia is more important than skeletal integrity, and that to minimize skeletal calcium storage, 1,25$(OH)_2$D not only increases calcium release from bone, but also stops calcium from being incorporated in bone (Lieben *et al.* 2012). Mice that survive weaning display inefficient clearing of the exogenous 1,25$(OH)_2$D compared to wild type mice, where hormone is eliminated between 6 and 12 hours. Interestingly, 25(OH)D serum levels are very low in *CYP24A1* deficient mice. Treatment with 25(OH)D does not result in 24,25(OH)D formation, as opposed to wild type mice. Also, both *VDR*-knockout mice and *CYP24A1*-knockout mice fail to catabolise 1,25$(OH)_2$D, indicating the importance of the 1,25$(OH)_2$D-inducible, VDR-mediated, C-24 oxidation pathway (Masuda *et al.* 2005).

## 1.4 Regulatory Processes - Endocrine Vitamin D and Mineral Homeostasis

### 1.4.1 Calcium and Phosphate Homeostasis

Minerals from the diet are essential for our survival. The process of their uptake and mineral "re-shuffling" is controlled by intestine, bone, kidney, parathyroid and their hormones 1,25$(OH)_2$D, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF-23) and calcitonin, which regulate the homeostasis. Most of them act via specific receptors, such as: 1,25$(OH)_2$D binds to VDR, calcium – calcium sensing

receptor (CaSR), PTH – PTH receptor (PTHr), FGF-23 – FGF receptor (FGFR) and co-factor Klotho, calcitonin – calcitonin receptor; specific receptor for phosphorous is unknown. Calcium and phosphorus are of particular importance – they both build our skeleton and create a buffer in extracellular fluids. Human body contains about 1000 grams (g) of calcium and about 600 g of phosphate. 99% of total calcium and 85% of total phosphorus is present in the skeleton in the form of hydroxyapatite (HAP) and crystalline, respectively. The rest - 1% of total calcium and 15% of total phosphate are present in extracellular fluid at saturation point (kept in solution by various molecules) and soft tissues acting as an essential buffer. Multiple cellular processes such as muscle contraction, relaxation, functioning of nervous system, cellular transport, blood clotting and many more are dependent on very tight regulation of free ionised calcium ($Ca^{2+}$: 1.13- 1.34 mM). Importantly, intracellular calcium can also act as a second messenger in many intracellular responses to chemical and electrical stimuli and required by various enzymes for full activity. The concentration of intracellular calcium is strictly controlled through action of calcium channels, calcium pumps and calcium exchangers. Ionised calcium is not distributed throughout the whole cell, but mostly localized to sarcoplasmatic reticulum in muscle, Golgi apparatus and mitochondria, which act as intracellular calcium stores. Intracellular heterogeneity of calcium, in form of calcium sparks or calcium waves is observed in a range of cells (myocardium, hepatocytes, oocytes and more) (Camacho and Lechleiter 1993, Cobbold *et al.* 1983, Pinton *et al.* 1998, Rooney *et al.* 1990, Takamatsu *et al.* 1991). Extraskeletal phosphate is present either in the form of inorganic phosphate ions or esters, serving as body's energy storage (inositol trisphosphate, cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate). Phosphate is also present in DNA, cell membranes' phospholipids

and is essential in signalling cascades where it acts as a regulator of protein function. Crucially, calcium and phosphorus product (Ca x $PO_4$) is maintained constant, and so for instance any decrease in calcium concentration results in an increase in phosphate ions.

#### *1.4.1.1 Calcium and the Hormones Involved*

The daily intake of calcium is about 1000 mg, which is comparable with consuming a litre of milk. Of that, approximately 300 mg is absorbed through the small intestine; 150 mg is secreted through the gut, giving a net absorption of 150 mg for a healthy adult (Barger-Lux *et al.* 1989, Mallette 1989, Yanagawa 1992). Ionised calcium in blood is under tight regulation of PTH, which is an 84-amino acid peptide with a half life of less than 4 minutes, that is synthesised by the chief cells of the parathyroid gland (Segre *et al.* 1974). When calcium concentration is high, PTH increases to counterbalance the changes. This negative feedback is mediated by the extracellular Calcium Sensing Receptor (CaSR), which belongs to the family of G-protein coupled receptors. The biological actions of PTH in regulating calcium homeostasis are exerted via binding to the PTH receptors and involve: stimulation of osteoclastic bone resorption and release of calcium and phosphate from bone, stimulation of reabsorption and inhibition of phosphate resorption from the renal tubules and stimulation of renal production of 1,25$(OH)_2$D, which in turn, increases intestinal absorption of calcium and phosphate.

Another hormone involved in calcium regulation is calcitonin – a 32-amino acid peptide, that is secreted by the parafollicular cells of the thyroid gland (Deftos 1993). Any increase in ionised calcium concentration induces secretion of calcitonin, which binds to calcitonin receptor and inhibits osteoclastic bone resorption via cyclic cAMP

pathway. Interestingly, aparathyroid patients do not experience alterations in calcium homeostasis, suggesting that this hormone may play a role in fine-tuning, i.e. additional but non-essential, rather than tight regulatory exerted by PTH.

Lastly, the steroid hormone 1,25$(OH)_2$D, as mentioned earlier is critical for calcium homeostasis. After hydroxylation in the liver, abundant 25(OH)D serves as a substrate for 1α-hydroxylation in the kidney to render the biologically potent form of the hormone -1,25$(OH)_2$D, with a half-life of about 5 hours. This renal 1α-hydroxyalation is the major control point in vitamin D metabolism and depends on local phosphate, calcium and PTH concentrations. When PTH and phosphate decrease, 1,25$(OH)_2$D synthesis is induced. It increases plasma calcium and phosphate concentrations by increasing the absorption of calcium from the intestinal tract. 1,25$(OH)_2$D acts on bone to increase calcium resorption and on the nephron by enhancing the effect of PTH, also to increase calcium reabsorption (Norman A. W. *et al.* 1982).

In healthy individual, the response to hypocalcaemia, i.e. a drop in ionised calcium concentration is instantly detected by the parathyroid glands, which respond by increasing the secretion of PTH. PTH increases osteoclastic bone resorption and, releasing calcium and phosphate from bone into extracellular fluid. At the same time, PTH also enhances renal absorption of calcium and inhibition of phosphate reabsorption. 1,25$(OH)_2$D is stimulated by the rise in PTH, effect of which is further increase in the absorption of the dietary calcium and phosphate in the gut *(Figure 1.7)*. The exact opposite is observed in hypercalcaemia, where increase in ionised calcium leads to decrease in PTH secretion and 1,25$(OH)_2$D synthesis, leading to increase in renal calcium secretion and decrease in intestinal calcium absorption. Increase in calcium concentration can also be detected directly by CaSR in the

kidney proximal tubule, which can then downregulate synthesis of $1,25(OH)_2D$ by inhibition of 1α-OHase (Bland *et al.* 1999, Kantham *et al.* 2009).

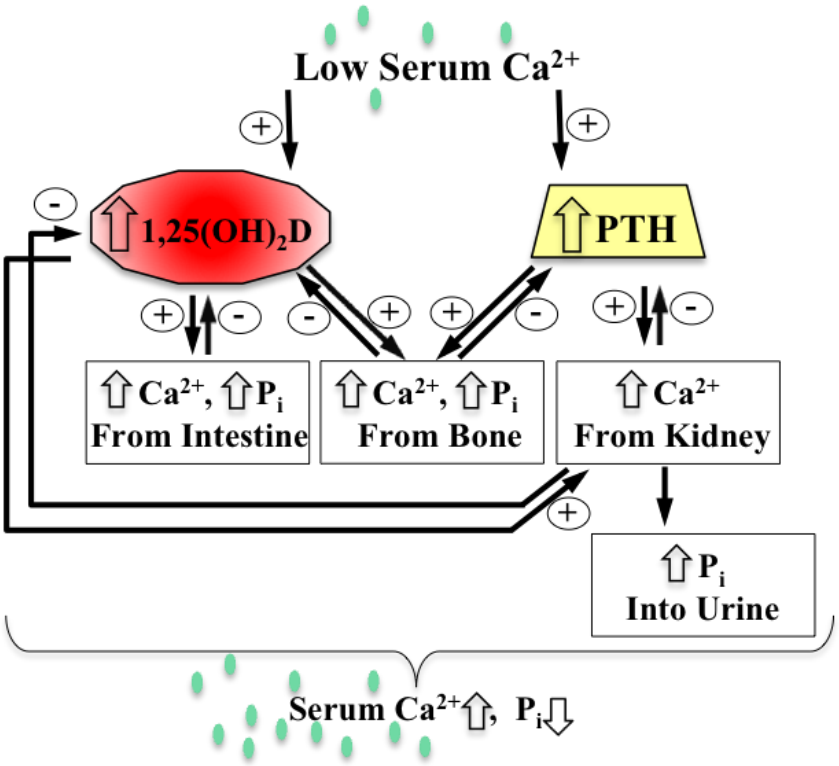


**Figure 1.7:** ***Hormonal response to hypocalcaemia leading to normocalcaemia****. $Ca^{2+}$ - ionised calcium, $P_i$ – phosphate. When concentration of calcium in the serum drops, PTH levels increase, which leads increased bone resorption and release of calcium and phosphate from bone into extracellular fluid. Raise in PTH also stimulates $1,25(OH)_2D$, which acts on the gut to increase calcium absorption.*

### *1.4.1.2 Phosphate and the Hormones Involved*

Similarly to calcium, the daily intake of phosphate is about 1000 mg; 80% is absorbed in duodenum and jejunum and filtered through the kidney (Murer *et al.* 2000). Renal proximal tubules are the major site of reabsorption of phosphate through three types of sodium dependent phosphate-co-transporters (Na-Pi-co-transporters): NaPi-IIa, NaPi-IIb, NaPi-IIc, also called NaPi2a, NaPi2b, NaPi2c, respectively (Coloso *et al.* 2003, Takeda *et al.* 1999). NaPi-IIa is expressed

exclusively in the proximal tubules, whereas NaPi-IIb is expressed predominantly in the small intestine's enterocytes. Regulation of transporters is exerted via actions of $1,25(OH)_2D$, PTH, calcitonin and indirectly via FGF-23 (Katai *et al.* 1999, Kempson 1996).

Two main hormones, which control phosphate homeostasis, are PTH secreted by the parathyroid and FGF-23 produced by osteocytes and osteoblasts in bone. In normal physiology, low serum phosphate leads to inhibition of FGF-23 and induction of $1,25(OH)_2D$, which increases phosphate uptake in the intestine and phosphate reabsorption from bone, and decrease in PTH, which facilitates phosphate uptake in the kidney *(Figure 1.8).* Conversely, high serum phosphate leads to increase in FGF-23 and decrease in PTH, which reduce expression of NaPi-IIa and Na-Pi-IIc in the proximal renal tubules, ultimately reducing phosphate reabsorption and increasing urinary phosphate excretion (Bergwitz and Juppner 2010, Gattineni and Baum 2010). Furthermore, FGF-23 suppress expression of 1α-OHase, leading to decrease in $1,25(OH)_2D$ levels in circulation, thus reducing intestinal calcium and phosphate absorption. There is also evidence that FGF-23 enhances the expression of *CYP24A1* mRNA (Quarles 2008, Shimada *et al.* 2004b).

FGF-23 is a 30 kDa protein with 251 amino acids, which signals via FGF receptor (FRFR1 to 5) and Klotho co-receptor, that converts canonical receptor for FGFs, FGFR1 into a specific FGF-23 receptor (Urakawa *et al.* 2006). Presence of FGF receptors has been demonstrated in parathyroid, bone, kidney and vasculature, as well as in other tissues (Ben-Dov *et al.* 2007, Lim *et al.* 2012). In parathyroid FGF-23 acts on the receptor complex to decrease *PTH* gene expression and PTH secretion by activation of MAPK pathway (Lavi-Moshayoff *et al.* 2010). In CKD both FGF-23 and PTH are increased, suggesting resistance of parathyroid glands to FGF-23. PTH

increases 1α-OHase in the kidney and consequently $1,25(OH)_2D$ synthesis. Recent evidence suggests that PTH induces expression and synthesis of FGF-23, which negatively controls $1,25(OH)_2D$ level (Hasegawa *et al.* 2010, Imanishi and Kawata 2005, Imanishi *et al.* 2012, Juppner 2011).

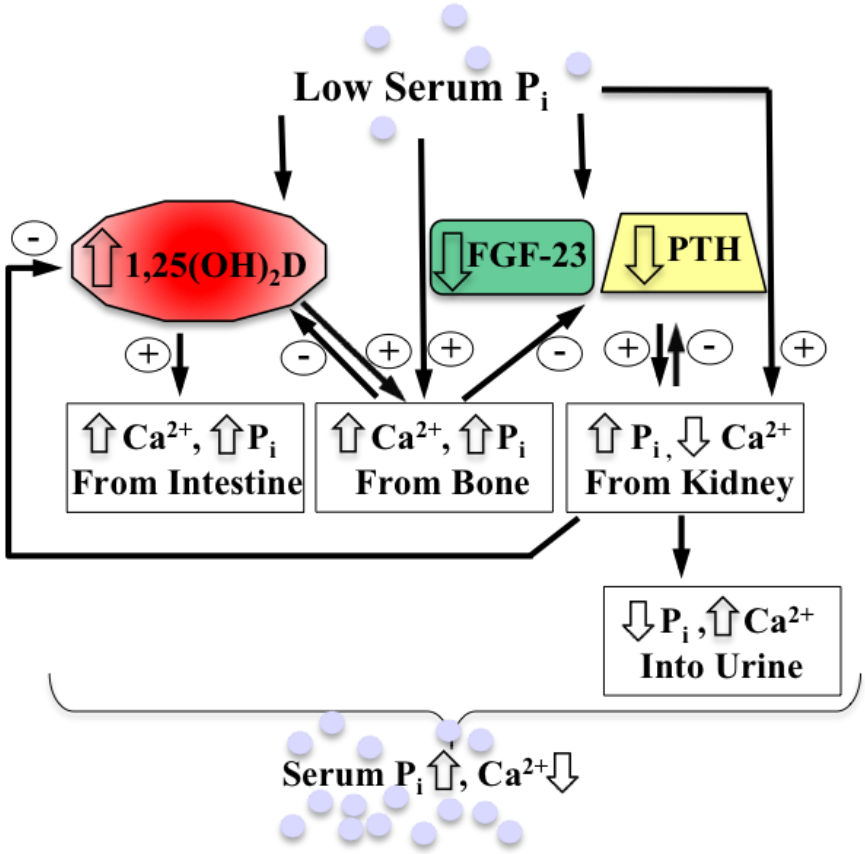


***Figure 1.8: Hormonal response to hypophosphataemia leading to normophosphataemia.*** *$Ca^{2+}$ - ionised calcium, $P_i$ – phosphate. When concentration of phosphate in serum drops, FGF-23 levels drop and $1,25(OH)_2D$ is induced. $1,25(OH)_2D$ increases phosphate uptake in the intestine and reabsorption from bone, whereas decrease in PTH facilitates phosphate uptake in the kidney.*

### 1.4.2 FGF-23 and Klotho – Novel Regulators of the Vitamin D System

FGF-23 and Klotho were studied separately until 2004, when Shimada and co-workers discovered that Klotho-deficient mice and FGF-23 deficient mice share the

same phenotype (Shimada *et al.* 2004a). Klotho was first identified over a decade ago, as a gene mutated in a mouse that developed premature aging syndrome and its name has been attributed to Klotho – a Greek goddess, who spins the thread of life (Kuro-o *et al.* 1997). The homozygous double *Klotho* mutant mice had elevated serum levels of $1,25(OH)_2D$ as well as increased calcium and phosphate (increased phosphate resembling change observed in CKD) (Yoshida T. *et al.* 2002). The *Klotho* gene is mapped to chromosome 13q12 and contains 5 exons and 4 introns. It encodes a single pass transmembrane protein with the large extracellular domain containing two repeats (KL1 and KL2 domains). It is a member of the 1 glycosidases protein family. Due to alternative splicing, several isoforms of Klotho have been identified. Membrane bound (mb) Klotho, which has a molecular weight of 130 kDa is thought to be critical for FGF-23 signaling by forming a ternary ligand-binding complex with FGF receptors, as described earlier.

To date, Klotho expression has been shown in distal renal tubules, proximal tubules, VSMCs and parathydoid glands (Komaba *et al.* 2010, Kuro-o 2009, Lim *et al.* 2012, Liu S. *et al.* 2008, Mian 1998). Recent evidence suggests that in addition to the membrane bound form, Klotho also exists in a soluble (s) form (where membrane bound protein is post-translationally cleaved by disintegrin and metalloproteinase (ADAM) family- ADAM10 and ADAM17, and released in a 130 kDa and 68 kDa Klotho fragments or it is generated through alternative splicing of exon 3 resulting in translation of 80 kDa protein), which has been detected in serum, urine and cerebro-spinal fluid (Imura *et al.* 2004, Matsumura *et al.* 1998, Saito *et al.* 1998, Wang Y. and Sun 2009). The role of sKlotho is different to the membrane-bound and involves regulation of the nitric oxide production in the endothelium, regulation of calcium homeostasis in the kidney as well as inhibition of intracellular insulin and insulin-

like growth factor-1 (IGF-1) signalling and Wnt signalling (Chang *et al.* 2005, Imura *et al.* 2007, Kurosu *et al.* 2005, Liu H. *et al.* 2007, Saito *et al.* 2000, Saito *et al.* 1998).

In vascular endothelial cells the sKlotho has been shown to inhibit TNF-α induced expression of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and vascular cell. In the kidney epithelial cells of distal convoluted tubule, the KL1 and KL2 domains of Klotho have been shown to have β-glucuronidase properties, which allow hydrolisation of the extracellular N-glycans on the calcium channel, transient receptor potential cation channel, subfamily V, member 5 (TRPV5). Cleavage of TRPV5 by Klotho entraps and activates the receptor at the plasma membrane modulating the intracellular calcium fluxes (Chang *et al.* 2005).

Suppression of insulin/IGF-1 signalling results in induction of mammalian forkhead box O (FOXO) transcription factors, which are negatively regulated by insulin/IGF-1 signalling. Conversely, activation of insulin/IGF-1 signalling induces phosphorylation and activation of serine-threonine kinase Akt, which inactivates FOXOs. Inactivation of insulin/IGF-1 signalling by Klotho allows FOXOs to bind to the promoters of anti-oxidant enzymes, including the mitochondrial manganese-superoxide dismutase (SOD2) and induce their mRNA expression (Wang Y. and Sun 2009). Therefore, it is not unreasonable to assume that Klotho may be directly or non-directly involved in the mitochondrial 1α-OHase or 24-OHase metabolism.

The Wnt signalling is essential for stem cell proliferation, however when chronically stimulated, it can lead to rapid stem cell apoptosis. Klotho's anti-aging properties are thought to be attributed to its ability to inhibit the Wnt signalling (Kuro-o 2008).

## 1.5 Paracrine -Autocrine Role of Vitamin D – The Non-Classical Effects

### 1.5.1 Expression and Regulation

Presence of extra-renal 1α-OHase was first documented in calvarial cells, then chondrocytes, keratinocytes and macrophages (Adams *et al.* 1983, Bikle D. D. *et al.* 1986, Pedrozo *et al.* 1999, Schwartz Z. *et al.* 1992, Turner *et al.* 1980). More recently in cells of the hematopoietic system, pancreas, placenta, parathyroid, prostate, and brain (Bland *et al.* 2004, Diaz L. *et al.* 2000b, Ritter *et al.* 2012, Somjen *et al.* 2007, Zehnder *et al.* 2001). Levels of locally produced $1,25(OH)_2D$ are difficult to measure, but it is known that disease-activated macrophages (e.g. tuberculosis) and placenta can produce high concentrations of $1,25(OH)_2D$, which can be detected in the circulation (Adams and Hewison 2012).

Regulation of the extra-renal vitamin D hormonal system, in particular 1α-OHase is a growing area of research. Evidence demonstrates, that the renal and extra-renal 1α-OHase is regulated differentially by the same stimuli. Below is the table compiling the results from a number of studies on regulation of 1α-OHase (it does not differentiate between mRNA, protein and activity) in kidney, macrophages, parathyroid and bone (*Table 1.1*).

***Table 1.1: Regulation of the extra-renal vitamin D 1α-Hydroxylase.*** *Parathyroid hormone (PTH), Fibroblast Growth Factor 23 (FGF-23), dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), Interferon gamma (INF-γ), Tumour Necrosis Factor alpha (TNF-α). Increase (↑), decrease (↓), *conflicting evidence. Results represent expression of either mRNA, protein or enzyme activity. (Anderson et al. 2003, 2005, Anderson et al. 2010, Bacchetta J. et al. 2012, Dunlay and Hruska 1990, Gao et al. 2002, Ito et al. 2012, Samadfam et al. 2008, Tang W. J. et al. 2010, Wohrle et al. 2011).*

| | 25(OH)D – 1α-Hydroxylase | | | |
|---|---|---|---|---|
| | Kidney | Macrophages | Parathyroid | Bone |
| **PTH** | ↑/No effect* | No effect | | No effect |
| **FGF-23** | ↓ | ↓ | ↑ | ↓ |
| **Calcium** | Low: ↑<br>High: ↓ | | High: ↑ | Low: ↑<br>High: ↑ |
| **Phosphate** | Low: ↑<br>High: ↓ | | | High:↑ |
| **$1,25(OH)_2D_3$** | ↓ | No effect | | No effect |
| **INF-γ** | No effect | ↑ | | |
| **TNF-α** | No effect | ↑ | | |

First significant difference concerns action of PTH - in kidney it was shown to stimulate *CYP27B1* expression (Dunlay and Hruska 1990, Gao *et al.* 2002) (conflicting evidence showed no effect of PTH on *CYP27B1*, suggesting that the enzyme is influenced by calcium, not directly by PTH (Anderson *et al.* 2003)). In macrophages PTH exerts no effect on *CYP27B1* mRNA, neither does $1,25(OH)_2D$, nor calcium - classical regulators of the endocrine vitamin D system. Interestingly, 1α-OHase activity was shown to be increased by inflammatory stimuli, such as INF-γ and TNF-α (Adams and Gacad 1985, Pryke *et al.* 1990, Stoffels *et al.* 2006). Increased concentration of calcium inhibits the kidney *CYP27B1*, but stimulates the expression of *CYP27B1* in parathyroid and bone. FGF-23 appears to not only

suppress renal 1α-OHase, as it was shown to exert the same effect in macrophages and bone. In parathyroid, on the other hand, FGF-23 stimulates the expression of *CYP27B1* (Anderson *et al.* 2003, 2005, Anderson *et al.* 2010, Bacchetta J. *et al.* 2012, Dunlay and Hruska 1990, Gao *et al.* 2002, Ito *et al.* 2012, Samadfam *et al.* 2008, Tang W. J. *et al.* 2010, Wohrle *et al.* 2011).

Similarly to 1α-OHase, the 24-OHase protein and mRNA is expressed in a variety of tissues, such as intestine, bone, parathyroid, skin and many more. It has also been found in colon, prostate and mammary cancers (Overbergh *et al.* 2000, Wood *et al.* 2004, Xie *et al.* 2002, Yang *et al.* 1999).

Nonetheless, the expression and regulation of extra-renal vitamin D hormonal systems will be addressed in more detail in individual results chapters.

### 1.5.2 Local Actions

$1,25(OH)_2D$ has been recognised to be involved in suppression of cell growth, regulation of apoptosis, control of differentiation in the skin and maintenance of healthy nervous system. It has also been shown to be an important regulator of the rennin-angiotensin system, insulin secretion in pancreas, angiogenesis, normal functioning of skeletal and heart muscle, cellular calcium homeostasis as well as an important modulator of both innate and adaptive immune responses.

#### *1.5.2.1 Proliferation and Differentiation*

VDR knockout studies on mice demonstrated increased colonic proliferation, as assessed by the proliferating cell nuclear antigen activity. Interestingly, heterozygous

*VDR*$^{+/-}$ mice exhibited a rate of proliferation that was intermediate between the knockout and wild type mice (Kallay *et al.* 2002). 1,25$(OH)_2$D has been shown to induce cell cycle dependent kinase inhibitor p21, which stops action of cyclin-dependent kinase 1 (CKD-1, responsible for cell cycle progression) in many cell types and to ultimately cause an arrest at transition from the post mitotic gap checkpoint (G0/1) to synthetic phase (S) (Moffatt *et al.* 2001).

Evidence shows that 1,25$(OH)_2$D is also important in differentiation of the cells from the haematopoietic system into macrophages – cells from VDR knockout mice did not differentiate into macrophages upon treatment with 1,25$(OH)_2$D, as opposed to the cells from the wild type animals treated in the same way (Gombart *et al.* 2006). Treatment of mouse keratinocytes with picomolar concentrations of 1,25$(OH)_2$D increased cellular proliferation, whereas higher -nano to micromolar concentrations of 1,25$(OH)_2$D stunned the growth entirely (Bollag *et al.* 1995). Interestingly, presence of 1α-OHase can also influence proliferation. It has been shown that when levels of 25(OH)D are too low to sufficiently activate VDR, cells expressing 1α-OHase reacted to environmental stress by suppressing their growth (Barreto *et al.* 2000). Conversely, cells lacking 1α-OHase did not exhibit such anti-proliferative action (Holick 2003). There is no evidence of how high the local tissue concentrations of extra-renal 1,25$(OH)_2$D can be, as they are almost impossible to measure. The locally produced 1,25$(O)_2$D can overspill to circulation or other tissues. In tuberculosis, bacterial infection triggers formation of aggregates of white blood cells in various organs. These white blood cells contain macrophages, which overproduce 1,25$(OH)_2$D, that in turn can overspill to circulation and surrounding tissue (Adams *et al.* 1983).

In bone basal *CYP24A1* mRNA levels are very low and stimulation with $1,25(OH)_2D$ leads to 400,000-fold induction. Also, in proliferating osteoblasts higher rate of $1,25(OH)_2D$ synthesis and, accordingly lower levels of 24-OHase have been reported (Anderson *et al.* 2003). It is possible that 24-OHase maintains low $1,25(OH)_2D$ levels during proliferating phase, to diminish hormone's antiproliferative activity, to then increase the levels of $1,25(OH)_2D$ during the mineralisation phase (Ylikomi *et al.* 2002). In other extra-renal tissues $1,25(OH)_2D$ has been shown not to have an inhibitory effect on the expression of keratinocyte 24-OHase, nor macrophage 24-OHase (Overbergh *et al.* 2000, Schuster *et al.* 2001, Xie *et al.* 2002).

The current knowledge in the area of local regulation of non-renal vitamin D system is not yet extensive. A consequence of which the research described in this thesis has been undertaken. What is known, is that $1,25(OH)_2D$ and VDR interact with various transcription factors and cell signalling systems, which often are cell-specific and which influence cellular growth. Evidence shows that $1,25(OH)_2D$ enhances expression of the androgen receptor and inhibits expression of the estrogen receptor (Zhao *et al.* 1999). It also binds to IGF-1 receptor and various IGF binding proteins in breast cancer cells (Demirpence *et al.* 1994). All of these actions can potentially induce p21 expression and growth inhibition (Boyle *et al.* 2001). Other studies, primarily on colon, prostate or breast cancers have shown that treatment with $1,25(OH)_2D_3$ decreases abundance of EGFR (colon) (Tong *et al.* 1998), or increases mRNA and protein of transforming growth factor β (TGFβ) (colon, breast, keratinocytes) which have antiproliferative effect (Chen A. *et al.* 2002, Colston and Hansen 2002).

$1,25(OH)_2D_3$ has also been shown to regulate angiogenesis i.e. the formation of new blood vessels from existing vasculature. Angiogenesis plays an important role in

tumour growth and metastasis, atherosclerosis, psoriasis and many other pathological conditions. Evidence from mouse model MCF-7 breast carcinoma studies indicates that $1,25(OH)_2D_3$ inhibits VEGF-induced endothelial cell proliferation, suggesting the potential use of $1,25(OH)_2D_3$ in both prevention and regression of pathological angiogenesis *(Mantell D. et al. 2000).*

### *1.5.2.2 Apoptosis*

Depending on the cell type, it has been reported that $1,25(OH)_2D$ can either inhibit or lead to cell death. In some colon cancer cell lines and retinoblastoma cells $1.25(OH)_2D$ was shown to upregulate the expression of pro-apoptotic B-cell lymphoma 2 associated X protein (Bax) and B-cell lymphoma 2 homologous antagonist **(**Bak), simultaneously downregulating the anti-apoptotic protein Bcl-2 and Bcl-XL (Beer *et al.* 2004, Diaz G. D. *et al.* 2000a, Wagner *et al.* 2003). Studies in breast cancer also demonstrated pro-apoptotic actions of $1,25(OH)_2D$, however via different mechanism – suppression of IGF receptors, upregulation of MAP kinase and inhibition of Akt kinase (Colston and Hansen 2002). On the other hand, in ovarian cancer $1,25(OH)_2D$ was shown to prevent the death receptor-mediated apoptosis (Zhang X. *et al.* 2005). This demonstrates the scope of $1,25(OH)_2D$ actions with respect to apoptosis may be vast and potentially depends on the cell specific receptors.

### *1.5.2.3 Inflammation*

$1,25(OH)_2D$ has been shown to enhance the phagocytic and chemotactic capacity of macrophages and to increase the pro-differentiating actions of monocytes, contributing to antibacterial and antiviral defences (Oh *et al.* 2009). Studies have

demonstrated that bacterial activation of Toll-like receptor 4 (TLR4) and Toll-like receptor heterodimers TLR2/1 in macrophages and monocytes results in upregulation of 1α-OHase, VDR and other VDR-target genes, and leads to induction of cathelicidin antimicrobial peptide (CAMP) and β-defensin 2 and killing of *Mycobacterium tuberculosis* (Liu P. T. *et al.* 2006). The CAMP promoter –*CAMP* and β-defensin 2 promoter *(HBD2)* have recently been shown to have NF-κB binding sites, which highlights the involvement of vitamin D signalling in the innate immunity (Liu P. T. *et al.* 2009). Another native mechanism of pathogen detection and elimination involves detection of the microbial structures by expression pattern recognition receptors. Recent studies confirmed that VDR can directly induce the expression of the gene encoding receptor NOD2/CARD15 (nucleotide oligomerisation domain protein 2/caspase recruitment domain containing protein 15). Disruption of NOD2 signalling has been associated with Crohn's disease, in which intestine becomes prone to microbial infection (Wang T. T. *et al.* 2010).

What is more, $1,25(OH)_2D$ was shown to inhibit the expression of co-stimulatory molecules (CD40, CD80, CD86) and major histocompatibility complex class II (MHC-II) on the cell surface of antigen presenting cells, ultimately leading to the inhibition of the production of inflammatory cytokines such as interleukin-12 (IL-12). Through this action, the cell's T-cell polarisation becomes shifted from a proinflammatory T helper 1 (Th1) to a protective T helper 2 (Th2) and regulatory T-cell phenotype. This is enhanced by $1,25(OH)_2D$'s ability to directly modulate T-cell responses, by inhibition of Th1 and Th17 cytokines and up-regulation of Th2 cytokines (Verstuyf *et al.* 2010).

1α-OHase and 24-OHase promoter activities are tightly regulated also in inflammatory environment to maintain high levels of 1,25(OH)D. In macrophages, a

mechanism, which allows the maintenance of high local concentrations of $1,25(OH)_2D$ involves action of Stat-1α (signal transducers and activators of transcription -1α) which is normally induced by INF-γ and acts to inhibit the transcription of 24-OHase (Vidal *et al.* 2002).

Cells of the immune system are a good model of auto/paracrine actions, as they are able to both respond and locally synthesise $1,25(OH)_2D$ (Stoffels *et al.* 2007, Stoffels *et al.* 2006). This process is regulated differently from the one in kidney cells, with immune stimuli rather than calcaemic stimuli regulating levels of $1,25(OH)_2D$ in inflammatory foci. Current evidence confirms that there are vast differences between regulation of renal and extra-renal 1α-OHase. For instance, 1α-OHase in sarcoid or cytokine activated macrophages, is not inhibited by $1,25(OH)_2D_3$, calcium or PTH (Adams and Gacad 1985, Dusso *et al.* 1997, Gates *et al.* 1986). Induction of 1α-OHase in these cells occurs by exposure to inflammatory stimuli, such as lipopolysacharide (LPS) or inflammatory mediators such as tumour necrosis factor alpha (TNF-α) or interferon gamma (INF-γ), often via NFκB signalling pathway. This suggests that the regulatory pathways involved in control of extra-renal 1α-OHase may be different to those characterised in kidney. Regulation of renal and extra-renal 1α-OHase will be addressed in more detail, with regard to calcium, phosphate, TNF-α, INF-γ, interleukin -6 (IL-6), interleukin 17A (IL-17A), FGF-23 and Klotho in the following results chapters.

#### *1.5.2.4 Regulation of Cellular Calcium*

Evidence shows that $1,25(OH)_2D$ is involved in modulation of cytoplasmic signalling in some cell types, such as osteoblasts, cells of parathyroid gland and cardiac myocytes. In osteoblastic ROS 17/2.8 cells, it has been demonstrated that

$1,25(OH)_2D$ can act rapidly to exert exocytosis which couples with intracellular calcium increase. This occurs partially via activation of L-type calcium channels. Intracellular calcium increase in these cells has been further reported to activate Akt signalling, which suppresses apoptosis (Xiaoyu *et al.* 2007). On the other hand, in parathyroid $1,25(OH)_2D$-induced elevation in intracellular calcium, activates the pro-apoptotic proteases, such as microcalpain and caspase 12 (Silver and Levi 2005). In cardiac myocytes, the rate and force of contraction is primarily mediated by the rate of calcium influx through calcium channels. VDR present in myocyte cell membrane has been shown to be involved in regulation of these calcium currents (Brette and Orchard 2003). 1,25(OH)2D induce the phosphorylation of protein kinase C (PKC) targets: phospholamban B and cardiac troponin I, which modulate intracellular calcium, accelerating muscle relaxation (Green *et al.* 2006).

### 1.5.3 Vitamin D and Vasculature

Data from microarray analyses revealed that the treatment of human bronchial and aortic SMCs with $1,25(OH_2)D$ induced upregulation in genes involved in morphogenesis, cell growth, survival and tissue remodelling (Bosse *et al.* 2007, Wu-Wong *et al.* 2006b, 2007b). VDR is present in VSMCs in a number of 200-25,000 copies per cell (Bouillon *et al.* 2008) and astonishingly it activates transcription of at least 103 genes in human, including plasminogen activator inhibitor-1 (PAI-1) and thrombospondin-1 (THBS1). It is also involved in down-regulation of the transcription of at least 78 genes, including those encoding PTH, PTHrP (autocrinely produced PTH related protein), and 1α-OHase (Wu-Wong *et al.* 2007b).

VSMCs respond to circulating levels of $1,25(OH)_2D$ and PTH, which stimulate increase in adenylate cyclase activity, macrophage chemoattractant protein-1 (MCP-

1), collagen, β-1 integrin, and cell proliferation. 1α-OHase involvement in a local synthesis of 1,25$(OH)_2$D and PTH levels in VSMCs, may be a protective mechanism from variations in circulating levels of these two hormones (Somjen *et al.* 2005), also the local synthesis can cater for the local requirements of 1,25$(OH)_2$D and disconnect the systemic calcaemic and phosphataemic effects from the local ones. Experimental evidence suggests that in VSMCs 1α-OHase is up-regulated in a dose dependent manner by estrogenic compounds, such as phytoestrogens and estradiol (Somjen *et al.* 2005).

## 1.6 Chronic Kidney Disease (CKD)

### 1.6.1 Definitions and Consequences

The kidneys are the body's main blood filtering organs, which regulate water and electrolyte content, but also act as endocrine glands. Through their ability to secrete hormones including 1,25$(OH)_2$D they can regulate calcium metabolism and bone homeostasis. The functional unit of a kidney is a nephron, with each kidney being made up of 400,000 – 800,000, this number is inversely correlated with age and kidney health (O'Callaghan 2009). One functioning kidney provides enough adequate renal tissue to have no negative implications on patient's health. However, in the case of a renal insult, tissue can become damaged and fibrose, which can subsequently lead to the development of chronic kidney disease (CKD) with the potential consequence of kidney failure (Longenecker *et al.* 2002).

The definition of CKD relates to the reduced renal function or kidney disease of any form that has been present for a period of time, from months to years. It can be a consequence of primary or secondary forms of glomerular, tubulo-interstitial or

vascular disease in the kidney (Mangione and Dal Canton 2010). It is a progressive and irreversible condition, which is commonly associated with old age, type 2 diabetes mellitus, hypertension and vascular disease. CKD is present throughout the world, with the prevalence reaching 15% in some countries, and still rising (O'Callaghan 2009). Progression of CKD in some patients may cause serious complications, resulting in the need for dialysis and kidney transplantation. The classification of CKD comprises of 5 stages *(Table 1.2)*, each of which refers to an estimated glomerular filtration rate (eGFR), the estimated rate at which the fluid and small molecules, primarily creatinine are filtered by the glomerulus from the plasma before being reabsorbed (Sarnak *et al.* 2003).

***Table 1.2***: ***Progression stages in CKD.*** *Adapted from (Levey et al. 2003).*

CHRONIC KIDNEY DISEASE PROGRESSION

| STAGE | eGFR* | DESCRIPTION |
|---|---|---|
| 1 | >90 | Normal or raised GFR + other evidence of kidney damage |
| 2 | 60-89 | Slightly reduced GFR + other evidence of kidney damage |
| 3A<br>3B | 45-59<br>30-44 | Moderately reduced GFR +/- other evidence of kidney damage |
| 4 | 15-29 | Severely reduced GFR +/- other evidence of kidney damage |
| 5 | <15 | Severely reduced GFR +/- other evidence of kidney damage |

** Measured in ml/min/1,73m²*

In the UK eGFR is calculated using Modification of Diet in Renal Disease (MDRD) formula, which is based on serum creatinine, age, race, gender (eGFR= 175 x Serum

Creatinine$^{-1.154}$ x Age$^{-0.203}$ x [1.212 if African American] x [0.742 if Female], measured in ml/min/1.73 m$^2$ (Levey *et al.* 1999). Stage 1 (eGFR>90) describes the mildest form, indicating onset of kidney disease, whereas stage 5 (eGFR<15) refers to most severe conditions, also described as ESRD, which often requires renal replacement therapy.

It is however important to note, that eGFR is somewhat a one-dimensional parameter, as it only reflects a single aspect of a deteriorating kidney function. In order to have a fuller picture, it is also important to account for the endocrine failure, which is reflected by decrease in VDR activation, erythropoietin synthesis failure, changes in PTH etc.

### 1.6.2 Clinical Impact – Mortality

Life expectancy of patients with CKD, compared with healthy individuals is significantly decreased *(Figure 1.9).*

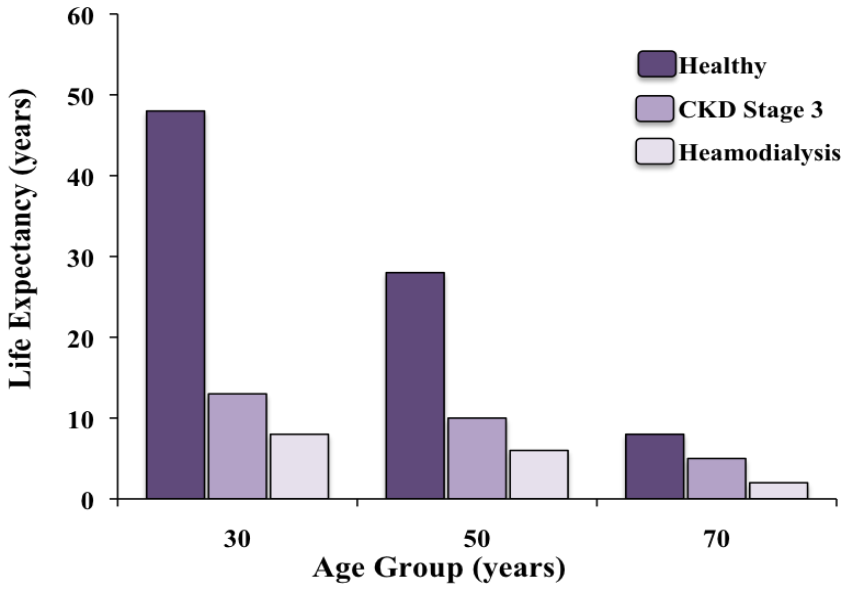


***Figure 1.9: Life expectancy across three age groups of patients with chronic kidney disease (CKD) Stage 3.*** *Haemodialised (HD) and healthy controls (Schell et al. 2013).*

Despite increased mortality at older age and at haemodialysis stage, CKD remains a killer in all age groups, leading to premature death even in children. CVD starts early in the course of CKD and is the most common cause of death in pre-dialysis, dialysis and post renal transplantation patients, including children with CKD (Go *et al.* 2004, Herzog *et al.* 2007, Shroff R. 2011). It has been reported that 50% of individuals suffering from ESRD die from CVD (Wang A. Y. *et al.* 2003).

CKD is more common among women than men and the likelihood of developing CKD increases with age (incidence of ESRD is higher in the adults over 65 years of age). Interestingly though, men are 50% more likely to progress to ESRD than women. Most important risk factors predisposing to CKD is diabetes, hypertension, CVD, obesity, high cholesterol and a family history of CKD. Acute kidney injury, infections, drugs or toxins can contribute to progression of CKD. In terms of ethnicity, Hispanics have 1.5 times the rate of kidney failure compared to non-Hispanic whites (CDC 2010). In UK, Asian population is more likely to develop diabetes and consequently CKD, compared to British whites (Kerr 2012).

### 1.6.3 Failure of the Vitamin D Hormonal System in CKD and its Consequences

#### *1.6.3.1 Endocrine Vitamin D Hormonal System*

The levels of $1,25(OH)_2D$ fall with loss of renal function, which usually starts early (eGFR of around 60 ml/min/1.73 $m^2$) resulting in high incidence of $1,25(OH)_2D$ deficiency in CKD. Defective $1,25(OH)_2D$ production contributes to the onset of secondary hyperparathyroidism (sHPTH), which is a marker of increased mortality rates in kidney disease. Kidney failure causes phosphate accumulation in the serum.

Further, in response to that plasma FGF-23 levels increase steadily, as the CKD progresses, also contributing to the failure of the normal functioning of the vitamin D hormonal system by suppression of 1α-OHase. Increased levels of FGF-23 are synthesized in bone. FGF-23 binding to FGF receptor-Klotho complexes enhances the excretion of phosphate in kidney. However, it also inhibits 1α-OHase and stimulates 24-OHase in kidney, leading to decreased serum blood $1,25(OH)_2D$ levels. Increase in FGF-23 leads to raise in PTH secretion by parathyroid, which results in the drop of $1,25(OH)_2D$ and consequently, hypocalcaemia (*Figure 1.10*).

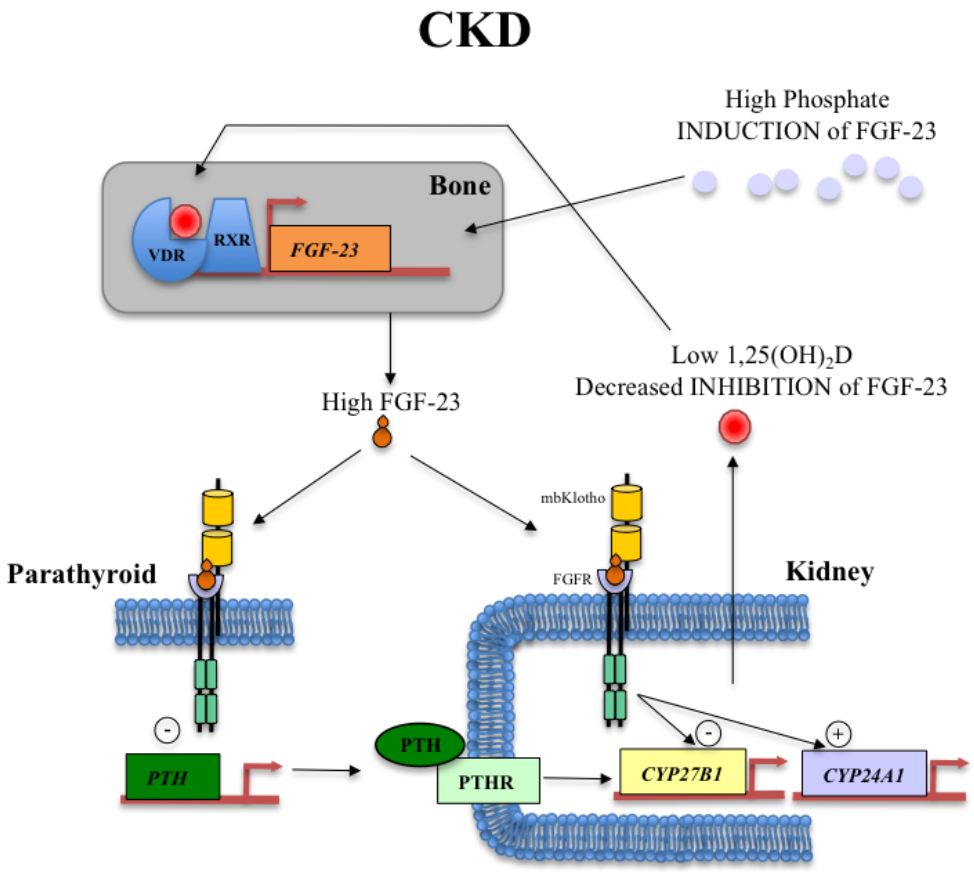


***Figure 1.10: Consequences of Chronic Kidney Disease (CKD) induced high levels of Fibroblast Growth Factor 23 (FGF-23) on vitamin D metabolism.*** *(+) induction, (-) inhibition, (PTHR) parathyroid hormone receptor, (CYP27B1) gene encoding vitamin D 1α-Hydroxylase, (CYP24A1) gene encoding vitamin D 24-Hydroxylase.*

As this process progresses, the bone-kidney-parathyroid axis becomes more disrupted, and resistant due to reduced tissue Klotho and also failure of the vitamin D hormonal system, which enhances hyperphosphataemia (Gutierrez *et al.* 2005, Hasegawa *et al.* 2010, Juppner 2011).

To confirm the direct effect of FGF-23, studies in rats with induced, early-stage CKD were performed, which have showed that exogenous administration of FGF-23 antibodies resulted in an increase in $1,25(OH)_2D$ levels, a normalization of serum calcium concentration and a decrease in PTH (Hasegawa *et al.* 2010)

Recent data suggest, that plasma concentrations of FGF-23 in ESRD can be elevated by several orders of magnitude (0-500 normal; 100 – 10,000 early stages of CKD; 10,000 – 100,000 ESRD) (Gutierrez *et al.* 2005, Isakova *et al.* 2009, van Husen *et al.* 2010). DMP1 (dentin matrix acidic phosphoprotein 1) is a negative regulator of FGF-23. In CKD, levels of DMP1 are high, suggesting that the protein function is either attenuated or that the FGF-23 becomes resistant to its action. High FGF-23 levels have been associated with CKD, LVH and mortality (Jean *et al.* 2009). Administration of oral phosphate binders to prevent intestinal absorption or long-acting PTH analogues have been proposed as potential ways of preventing CKD-related FGF-23 increase (Juppner 2011).

#### *1.6.3.2 Paracrine/Autocrine Vitamin D Hormonal System*

In CKD, local activity of 1α-OHase is attenuated and this results in failure of local production of $1,25(OH)_2D$. This may be attributed to high phosphate load, decreased numbers of nephrons induced by necrosis or apoptosis, as well as increased concentrations of FGF-23 in the serum of CKD patients (Coburn J W 1994, Portale *et al.* 1984, Zhang M. Y. *et al.* 2002).

## 1.7 Artery

### 1.7.1 Normal Function

Arteries are muscular and elastic tubes that transport blood under a high pressure exerted by the pumping action of the heart. By contraction (systole) and relaxation (diastole) of the heart vascular cells change the luminal diameter, which allows blood vessels to maintain a pulse pressure to transform a pulsating blood flow into a continuous flow for the peripheral tissue. During diastole, the pulse reflection allows a flow-back into the coronary arteries. Systemic arteries carry blood from the heart through the smaller arterioles and capillaries to the rest of the body, whereas pulmonary arteries carry blood from the heart to the lungs.

Arteries are composed of three main layers: external layer of connective tissue and elastic membrane called *tunica adventitia*, followed by the layer of SMCs – *tunica media* and internally located monolayer of endothelial cells resting on connective tissue and internal elastic membrane – *tunica intima (Figure 1.11A).*

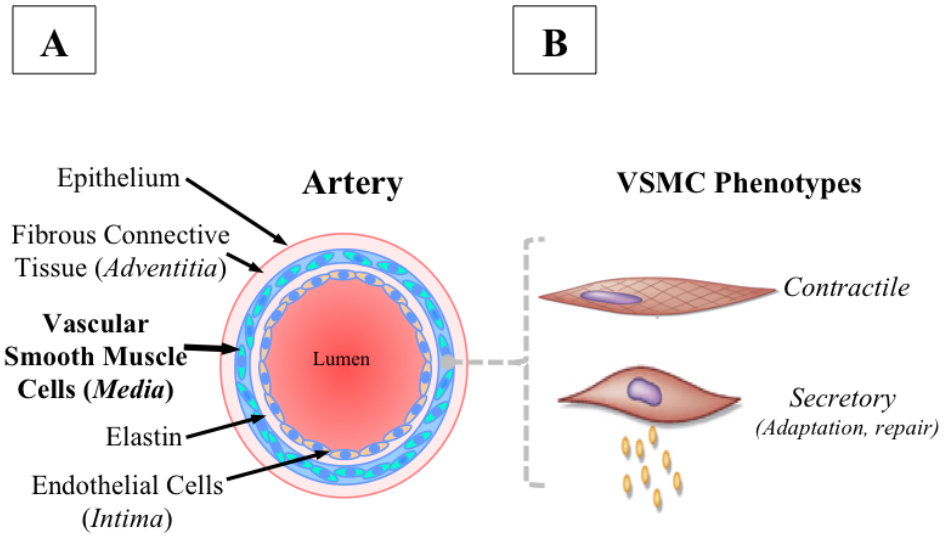


***Figure 1.11: Cross-section of normal artery and schematic representation of vascular smooth muscle cell (VSMC) phenotypes present in the media.*** *(A) Schematic representation of the layers of artery. (B) Different phenotypes of VSMC present in the media.*

Systemic arteries are divided into two main types – muscular, which tend to be larger (>10 mm in diameter), and elastic, which tend to be smaller (0.1 – 10 mm in diameter) with corresponding differences in the proportion of these layers. Aorta, for instance is the main systemic artery, which is connected directly to the heart (approximately 30 mm in diameter) (Hager *et al.* 2002) It acts as an elastic buffering chamber behind the heart, modulating wall elasticity to regulate compliance (the Windkessel function) (Belz 1995). It branches out to form coronary arteries, which branch further out into the brachiocephalic artery, left common carotid and the left subcalvian arteries. Renal arteries (*arteria renalis*) are muscular and branch out of the abdominal aorta and have diameter of about 25 mm (Turba *et al.* 2009). The role of muscular arteries and arterioles is to regulate the blood reflection, distribution and conductance.

#### *1.7.1.1 Structural Components of the Artery and Their Role in Adaptation, Protection and Repair*

##### *1.7.1.1.1 Endothelium*

Since endothelium is the most inner layer of the artery, it is submitted to continuous shearing forces of the circulating blood. It also is in constant contact with circulating cells and plasma components. Endothelial cells are smooth and elongated, contain myofibrils and renew every 2-6 years (Tedgui 1999). Their luminal surface is coated with glycoprotein coat forming glycocalix responsible for the anti-thrombogenic properties of this surface. ABO antigens, factor VIII antigen, and many others give endothelial cells distinctive immunologic characteristics (Tedgui 1999). Endothelial cells are packed tightly, connected by tight junctions forming *zona occludens*, which provides a tight seal, allowing 1-10% of luminal protein to penetrate into the wall

(Simionescu *et al.* 1976, Tedgui 1999). The movement of a material from the lumen into the vessel wall occurs via abundant pynocitic vesicles.

#### *1.7.1.1.2 Tunica Media and Smooth Muscle Cells*

The function of the medial layer of the artery is generation of force for vasoconstriction. This is due to the presence of thick layer of SMCs, embedded in the mesh of basal lamina and collagen fibrils, tied to a system of elastic fibrils. Factors that influence contraction and relaxation of SMCs are G protein-coupled receptors' modulators, pressure, tension, agents acting on ion channels or signalling systems, growth factors and extracellular matrix components, cell adhesion molecules and integrins (Hunt 2002).

There are many different phenotypes of VSMC, with contractile and synthetic representing two most distinct morphologies. Interestingly, the vastness of phenotypes translates into the diversity of functions *(Figure 1.11B).* What is more, VSMCs have a remarkable capacity to switch between phenotypes, also referred to as 'phenotypic modulation' (Hao *et al.* 2003). The main purpose of such biological plasticity is adaptation and repair. The contraction of VSMCs is involuntary and is regulated by the cytosolic calcium concentration, as well as the sensitivity of calcium of the contractile elements reacting to changes in the cell-surrounding environment. The contractile state is maintained by binding of hormones, neurotransmitters such as adrenaline and other auto/paracrine chemical signals to their receptors on the cell. The contraction then involves calcium influx to the cell, which is exerted via multiple mechanisms. The next step involves calmodulin binding and activation of myosin light chain kinase (MLCK), with calmodulin-MLCK complex catalysing phosphorylation of myosin light chains enabling actin-myosin interaction and force

generation. α-actin is one of the most abundant proteins of the VSMC cytoskeleton, hence why it is often used as an endogenous indicator of phenotype (Hunt 2002). Healthy contractile phenotype of VSMCs is characterised by abundant proteins involved in the process of contraction, such as α-actin, desmin and vimentin. Also cell morphology has a characteristic elongated, spindle like shape. During transition to secretory phenotype cells become more rounded and irregular in shape. Progressive changes in the cytoskeleton result in reduction of α-actin and expression of specific transcription factors. Secretory phenotype is a response to adaptation and/or repair – cells are capable of releasing matrix vesicles containing minerals or other unwanted compounds into the ECM in order to maintain intracellular homeostasis (Thyberg *et al.* 1997).

#### *1.7.1.1.3 Adventitia*

The cells of the adventitia are sparse and the majority are fibroblasts. The supply of oxygen and nutrients to adventitia and media is facilitated by a network of microvasculature - *vasa vasorum* (present only in larger arteries) (Heistad *et al.* 1981). Adventitia also contains nerves, which regulate SMCs function; lymphatic network and perivascular connective tissue. Pericytes have enormous differentiation capabilities and depending on the need they can become fibroblasts, SMCs or macrophages. They are important in angiogenesis and have been implicated in the regulation of the blood flow (Peppiatt *et al.* 2006).

### 1.7.2 Normal Regulation

Arteries have a natural capacity to regulate lumen diameter in response to changes in the blood flow. Vascular endothelium plays an important role in regulation of

vascular homeostasis. One of essential functions of the endothelium is the synthesis and release of the vasodilator nitric oxide (NO) (Ignarro *et al.* 1987). NO is synthesized from the amino acid substrate L-arginine by the enzyme endothelial nitric oxide synthase (eNOS) (Palmer *et al.* 1988). In response to mechanical shear stress or binding of acetylcholine or bradykinin to their receptors, NO diffuses through the basal membrane to SMCs, where it initiates conversion of GTP to cyclic GMP through soluble guanylyl cyclase orchestrating VSMC relaxation, vasodilation, proliferation and permeability (Moncada 1993, Scott-Burden *et al.* 1993).

### 1.7.3 Pathological Processes

Chronic stress on vasculature can be caused by diabetes, smoking, infection, CKD. In principle, it can be divided into localised (effect of which may be atherosclerosis – formation of atheroma, localised fat and cholesterol containing plaque in the intimal layer of the artery) and systemic (arteriosclerosis – loss of elasticity and consequently hardening of the arteries due to prolonged blood overload occurs typically in old age).

Both atherosclerotic and arteriosclerotic changes may lead to calcification. Progression of intimal calcification can result in plaque dislodgement causing myocardial infarction, i.e. downstream occlusion of the artery. Small areas of calcifications with sparse calcium phosphate crystals seem to be more bioactive than larger calcification plaques, resulting in triggering an inflammatory response and eventually cell death (Ewence *et al.* 2008)

Eutrophic inward remodelling is the physiological response to raised blood pressure. However, if the high blood pressure overpowers arteries' ability to autoregulate, then a blood vessel, which is exposed to high stress, switches to replace eutrophic inward

remodelling with hypertrophy. Important modulators of tissue remodelling are matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs). MMPs are collagenases with the ability to degrade polymerised, supramolecular collagen, that has been organised into fibrils (type I, II, III).

## 1.8 Artery and CKD

### 1.8.1 CKD and Consequences to Arteries and Heart

Abnormal changes in the structure and function of the vasculature begin very early in the course of renal decline, regardless of age (Shroff R. 2009). As kidney function reduces, the balance between salts and fluid alters affecting blood pressure. Increased blood pressure consequently contributes to higher tension of the artery wall. Further, the disrupted mineral homeostasis, i.e. lower capacity of maintaining minerals in solution contributes to ectopic soft tissue calcifications. Hormonal failure in CKD may be further exacerbated by local stressors, such as anaemia, hypoxia, low levels of circulating $1,25(OH)_2D$ and increased inflammation.

In hypertension and chronic uraemia, arterial stiffness increases blood velocity, causing vessel constriction and triggering myogenic response, which involves tissue remodelling, especially in smaller arteries (Khavandi *et al.* 2009). The signalling pathways involve modulation of transient receptor potential (TRP)-like channels (located mostly on plasma membrane, non-selectively permeable to cations such as sodium magnesium and calcium), changes in levels of diacylglycerol and protein tyrosine kinases, but also changes to intracellular calcium (Hill *et al.* 2006, Khavandi *et al.* 2009).

A study by Luksha (Luksha *et al.* 2011) analysed changes in peripheral arteries of ESRD patients undergoing haemodialysis. The group reported no NO contribution to flow, increase in asymmetrical dimethyl-L arginine (ADMA; inhibitor of NO, also referred to as 'a ureamic toxin') high expression of heat shock protein 70 and 27, no arterial remodelling and reduced arterial distensibility reflecting passive properties of the vascular wall. Loss of elasticity is also caused by the elastic fibre defragmentation and may be triggered by the arterial insults mentioned above, as well as a reduction in local and systemic calcification inhibitors, such as fetuin A, albumin and osteopontin (OPN), leading to pathological arterial stiffness (Schoppet *et al.* 2008) (Chung *et al.* 2009, Van Herck *et al.* 2009). Furthermore, it is thought that changes in *vasa vasorum* branching pattern, observed in arteriosclerosis may further contribute to vascular dysfunction (Mulligan-Kehoe 2010).

Uraemia is an accumulation of waste products, normally excreted in the urine (urea, creatinin, phosphate) in the circulating blood, as a result of a diminished kidneys' filtration ability in CKD patients. This is often observed together with low haemoglobin levels, low to normal calcium levels and abnormally high cytokine levels in the blood. Overall, uraemia associated alterations are observed mainly in high circulating volume and increased salt content in the blood. These biochemical and physical changes initiate development of arteriopathies, which are further aggravated by the ingress of cholesterol into the vessel wall as well as oxidation and modification of LDL cholesterol, mineral imbalance.

What is more, ESRD is characterised by presence of low-grade systemic inflammation, which also causes damage to vasculature. Inflammation induced pathological changes within arteries are characterised by the expression of adhesion molecules, recruitment of inflammatory cells, activation and migration of VSMCs

and secretion of calcium pyrophosphate crystals. Importantly, in uraemia, changes also occur as a result of a disrupted endocrine hormonal system at two levels – first, being a result of the vasculature insult itself and second, being a consequence of an endocrine imbalance caused by kidney pathology in CKD. Taken together, all the insults to the arteries accompanied by decreased oxygen levels, which are common in uremic patients contribute towards structural changes in vasculature. Left ventricular hypertrophy (LVH), decreased capillary density, progressive cardiac fibrosis and osteoblastic transformation of VSMCs, all ultimately link with alterations in cell function, such as mitochondrial dysfunction, apoptosis, changes in extracellular matrix, build up of bone-forming proteins, eventually resulting in myocardial infarction (Talmor *et al.* 2008) (Schiffrin *et al.* 2007, Yerkey *et al.* 2004) (McCullough *et al.* 2008).

### 1.8.2 Vascular Calcification and its Contribution to the Loss of Arterial Elasticity

Calciphylaxis is a calcification of skin arterioles, an extreme form of medial calcification, which can occur as a consequence of dialysis. It is very rare (only when the skin barrier function is damaged and can be breached) complication of dialysis can ultimately cause local tissue death and lead to severe infections. Medial calcification of the peripheral arteries can be detected by increased pulse wave velocity, high cardiac load and lowered perfusion (London *et al.* 2003). Evidence suggests that in patients undergoing dialysis, changes are even more pronounced, as calcification can be present in the heart valves and even the myocardium. Widespread calcification leads to hardening of the arteries, which increases afterload and end-systolic left ventricular pressure during every heartbeat through decreasing

aortic compliance and premature repercussion of the systolic pulse wave. Taken together, these changes lead to ventricular dysfunction and predispose to sudden cardiac arrest (Shanahan 2006) (Merx *et al.* 2005). As mentioned before a known VC inhibitor Fetuin-A is an important player and patients on dialysis exhibit low serum levels of this crucial protein (Wang A. Y. *et al.* 2003). *In vitro* studies in Fetuin-A knockout mice, which spontaneously develop soft tissue calcifications, demonstrated that compared to wild type the myocardial calcium content increased by 60% and was associated with strong induction of profibrotic TGF-β as well as downstream collagen and fibronecting mRNA synthesis (Merx *et al.* 2005).

### 1.8.3 What happens -Vascular Fibrosis and Calcification

VSMCs play a crucial role in the pathogenesis of VC. The mechanism of VC in uremic milieu is complex and not fully understood. It is unclear whether intimal and medial forms of calcification are identical or if they have different inciting factors leading to the same common pathogenic process, which parallels bone formation (Moe and Chen 2008). Until recently it has been considered to be an entirely passive unregulated process occurring due to plasma supersaturated with Ca and Pi, resulting in accumulation of Ca x Pi product (crystals of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], carbonated hydroxyapatite and amorphous calcium phosphate), mainly released by VSMCs and pericytes, enclosed in pinching-off vesicles (Reynolds *et al.* 2004) (Tanimura *et al.* 1986). So far, evidence demonstrates that the processes leading to increased calcification triggers transformation of VSMCs to an osteoblastic or chondrocytic phenotype. However, it has been argued that the transformation itself may be the cause of VC (Shanahan *et al.* 1999, Tyson *et al.* 2003). Which of these is the primary event remains inconclusive. Interestingly, the bone proteins, such as

runt-related transcription factor 2 (RUNX-2), osteonectin (OSN), osteopontin (OPN), bone sialoprotein, type I collagen, alkaline phosphatase (ALP) and sclerostin have also been identified in extra skeletal calcifications, indicating that tissues other than bone, in pathological conditions may resemble bone phenotype (Reynolds *et al.* 2004, Schoppet *et al.* 2008, Thambiah *et al.* 2012, Zhu *et al.* 2011).

#### *1.8.3.1 The Mechanism of Vascular Calcification in VSMCs*

Despite superaturation of all extracellular fluids with calcium and phosphate, in normal physiology, tissue mineralisation only occurs in bone. Bone mineralisation is a highly regulated process, which is initiated by hypertrophic chondrocytes releasing small matrix vesicles. The microenvironment of such vesicles allows nucleation of hydroxyapatite (HAP) crystals. The bone matrix is then mineralised by action of multiple mineralisation-regulating proteins. This phenomenon of selective mineralisation is attributed to the powerful mechanism of calcification inhibitors.

Normal VSMCs, do not calcify when exposed to supraphysiological concentrations of calcium and /or phosphate in vitro for prolonged periods of time. Interestingly though, VSMCs from CKD patients (both pre-dialysis and dialysis) are hugely prone to calcification. It is thought that VSMCs which were previously exposed to CKD milieu, whereas *in vivo* or *in vitro,* incur phenotypic changes and become primed for calcification, when further exposed to high calcium and phosphate concentrations (Shroff R. C. *et al.* 2010). One of the phenotypic adaptations of CKD VSMCs is a production of matrix vesicles containing calcium, phosphate and their product HAP crystals. Vesicles are released from both normal and apoptotic cells as a stress response directed to eliminate excess of intracellular calcium or in response to other

damage (aneurysms, hypertension, atherosclerosis, 1,25$(OH)_2$D toxicity). Body's natural response to presence of calcium-loaded vesicles excreted from VSMCs is phagocytosis. If however phagocytosis is impaired in CKD, calcification may be exacerbated. Interestingly, in absence of supraphysiological calcium and phosphate, *in vitro* studies have demonstrated that vesicles are loaded with mineralisation inhibitors, such as Matix Gla protein (MPG) and fetuin –A which deprive vesicles of mineralising properties. When the balance between inhibitors and inducers of VC is altered, vesicles can acquire mineralising properties.

Evidence shows that VSMCs are capable of producing the same transcription factors and proteins as bone, and can be induced to do so with high concentrations of phosphorus, uremic serum, high glucose, oxidized lipids, cytokines, and several other factors (Moe and Chen 2008).

Transformation of VSMCs into osteoblast-like cells is an active process, progression of which is dictated by the balance between inhibitors and inducers on a local and systemic level, in presence of systemic changes such as high PTH and low 1,25$(OH)_2$D (Jono *et al.* 2000, Reynolds *et al.* 2004). The simple model of the mechanism of VC is illustrated in *Figure 1.12.*

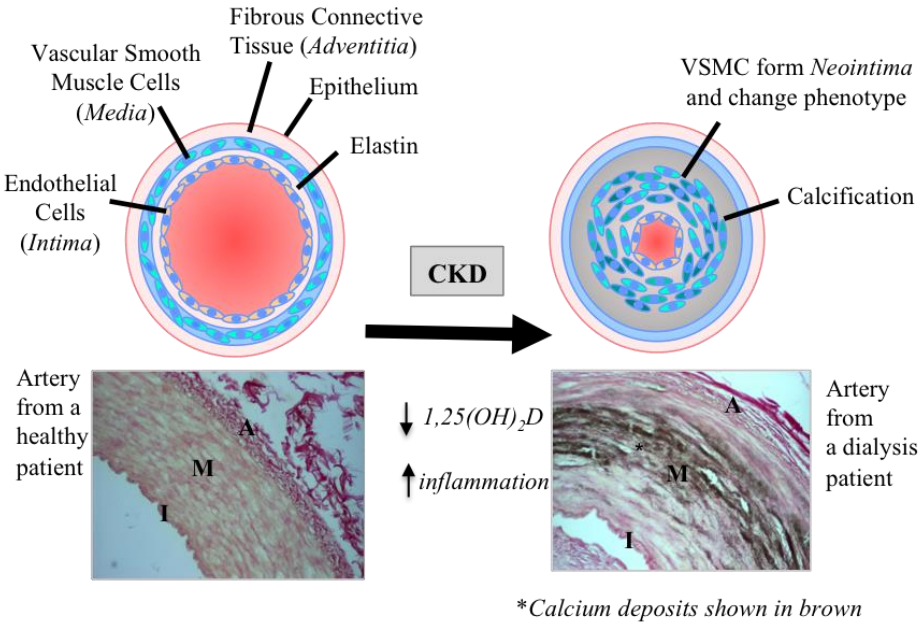


***Figure 1.12: The mechanism of Vascular Calcification in Chronic Kidney Disease (CKD).*** *Graphic representation of the phenotypic changes within an artery, as CKD progresses. Histology: human artery stained with Alizarin Red; abundant calcium deposits (brown) localize to medial (M) layer of the artery, only in patient undergoing dialysis. Intima (I), Adventitia (A).*

According to some reports, phosphate, which extracellular concentrations are increased in CKD appears to be one of the most important inducers (Wu-Wong *et al.* 2006c). These high levels of phosphate cause phosphate influx into the cell via the activation of type III sodium dependent phosphate co-transporters (Pit-1). This might be the trigger initiating the osteogenic process in VSMCs confirmed by the up regulation of RUNX-2 and the regulation of bone associated proteins such as osteopontin and osteocalcin. Others reported that calcium is a more powerful calcification inducer than phosphate, as for the fixed calcium and phosphate concentrations, increased calcium was more potent stimulus to induce calcification (Reynolds *et al.* 2004, Shroff G. R. *et al.* 2004). Osteopontin, osteoprotegrin, MGP, Smad-1 and nucleotide pyrophosphatase phosphodiesterase-1 (NPP-1) are known endogenous inhibitors of VC in VSMCs.

Fetuin-A and $1,25(OH)_2D$ (the latter via a direct effect) are thought to be important inhibitors of vascular calcification both locally and systemically and progression of CKD correlates with their low serum level. It is important to note that an indirect effect of $1,25(OH)_2D$, through increased absorption of calcium may lead to an increase in VC (Ketteler 2005). The VSMC endogenous regulators of VC and those present in the circulation have been summarised on the following page *(Figure 1.13) (Bucay et al. 1998, Hruska et al. 2005, Johnson et al. 2005, Jono et al. 2000, Kendrick and Chonchol 2011, Ketteler et al. 2003, Luo et al. 1997, Schoppet and Shanahan 2008, Schoppet et al. 2008).*

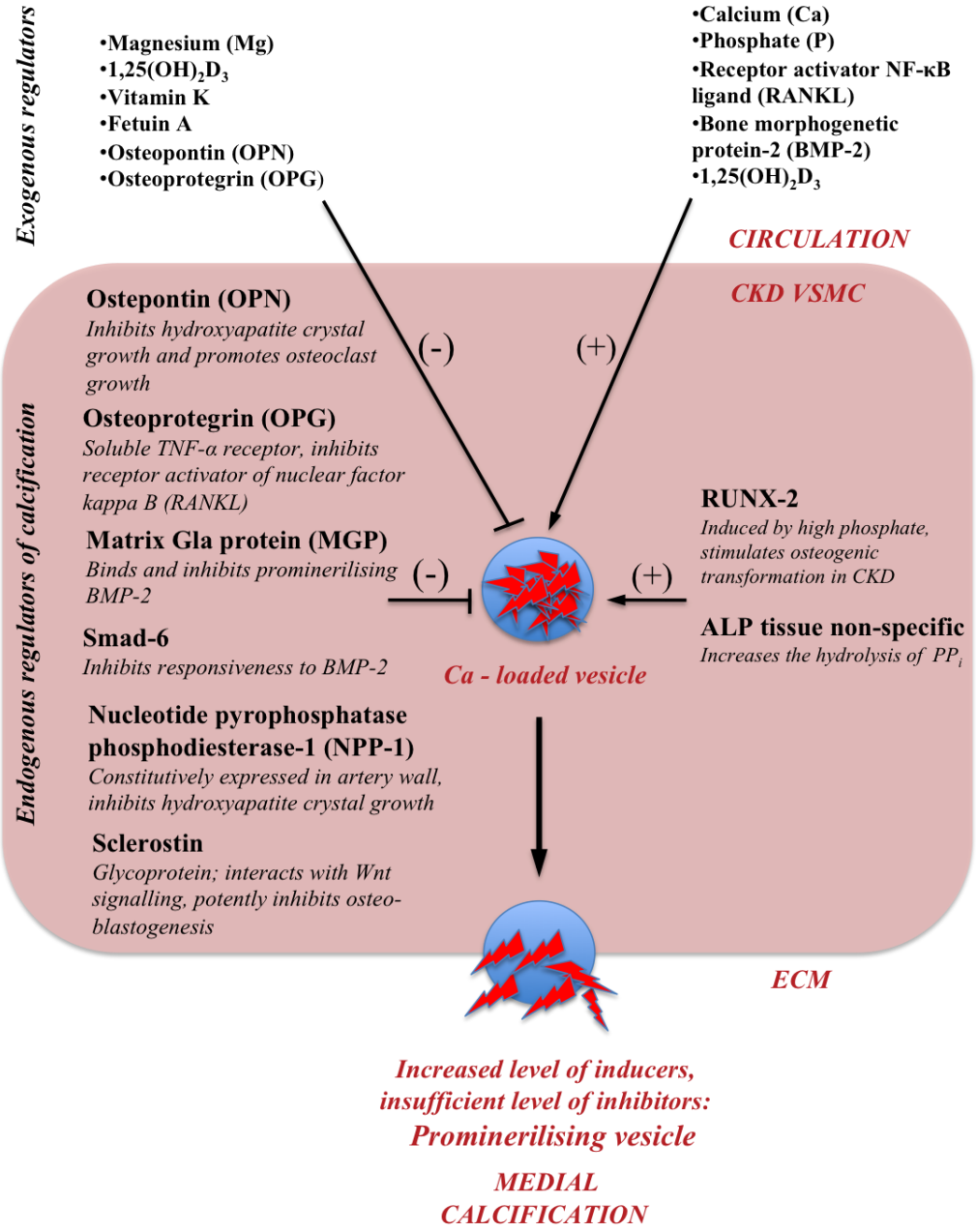


***Figure 1.13: Endogenous (VSMC) and circulating regulators of vascular calcification (VC).*** *$PP_i$ – pyrophosphate (Bucay et al. 1998, Hruska et al. 2005, Johnson et al. 2005, Jono et al. 2000, Kendrick and Chonchol 2011, Ketteler et al. 2003, Luo et al. 1997, Schoppet and Shanahan 2008, Schoppet et al. 2008).*

### 1.8.4 $1,25(OH)_2D$ and Vasculature

Newly emerged data from microarray analyses revealed that the treatment of human bronchial and aortic SMCs with $1,25(OH_2)D$ induced upregulation in genes involved

in morphogenesis, cell growth, survival and tissue remodelling (Bosse *et al.* 2007, Wu-Wong *et al.* 2006b, 2007b). Failure of cardiovascular VDR activation results in hypertension, cardiac hypertrophy, accelerated atherosclerosis with vascular calcification and renal failure. $1,25(OH)_2D$ is VDR's natural ligand. However, a spectrum of selective and non-selective analogues has been discovered in recent years. VDRA (VDR activator or agonist: paricalcitol, doxercalciferol and many more) therapy mainly involves suppressive effect of the parathyroid gland and regulation of calcium and phosphorus absorption in the intestine and mobilization in bone (Andress 2007). This, however, in clinical reality is not free of side effects. Non-classical VDR activation (i.e. with the use of analogues or in the local tissues) is a fascinating concept and we are just starting to deepen our understanding in this area. Various different proteins are involved in the VDR activation and different VDR activators can evoke different effects depending on the cell type and tissue. Despite the fact that the same gene is present in all body cells, when $1,25(OH)_2D$ binds to VDR in a proximal tubule cell, the proteins recruited in the transcription process vary from the ones present in, for instance, VSMC.

Recent studies on ApoE mice, an established model of atherosclerosis, show that the treatment with $1,25(OH)_2D_3$ and its less calceamic analogue paricalcitol resulted in elevation of VDR expression in aorta. However, the dose dependent study further showed that paricalcitol reduced TGF-β expression in plaques, as opposed to $1,25(OH)_2D_3$, which additionally contributed to significant VC and increase in expression of bone related proteins (Becker *et al.* 2011).

### 1.8.5 Therapeutic Concepts: Vitamin D - Active Metabolite

The first treatment for CKD, in 1970s, was with aluminium. A decade later routinely used therapeutic agent was calcium carbonate, followed by calcium acetate few years later. The action exerted by these compounds was binding to phosphate, aiding its excretion and reducing abnormally high levels. In the late 1990s, calcitriol became the treatment of choice for CKD stage 3 to 5, which significantly improved survival in CKD patients (Brown and Slatopolsky 2007, Kovesdy *et al.* 2008a).

Since plasma concentrations of circulating $1,25(OH)_2D$ are 1000 fold lower than those of 25(OH)D, with the half life of the active metabolite being substantially lower, the preferred metabolite measured in assays is the latter. The optimal concentration of 25(OH)D ranges between 40 and 120 nM. This wide range causes confusion and is an effect of differences in endpoints (fractures versus serum PTH) and differences in the analytical methods measuring serum 25(OH)D (Dawson-Hughes *et al.* 2005).

The therapeutic concept of administration of an active metabolite came from understanding the molecular mechanism of vitamin D bio-activation by 1α-OHase, mainly in the kidney. In CKD, levels of this essential enzyme are significantly reduced impairing the bio-activation process. This is likely due to the uraemic mineral and metabolic imbalance and consequently tissue apoptosis/necrosis.

### 1.8.6 Vitamin D Therapy versus Cardiovascular Complications and Patient Mortality

In humans, there are well-documented benefits of active vitamin D therapy, such as control of secondary HPT, cure of osteomalacia and osteitis fibrosa cystica, demonstrated in studies using bone biopsies (Dusso *et al.* 2005, Olgaard and Lewin

2006). What is more, the recent study by Kovesdy compared the association between mortality and calcitriol treatment in patients undergoing and not undergoing dialysis (CKD stages 3-5) and concluded that therapy with $1,25(OH)_2D$ significantly improved survival of non-dialysis patients (Kovesdy *et al.* 2008b).

The safe correction of 25(OH)D and $1,25(OH)_2D$ deficiency are essential in improvement of the outcomes in CKD (Dusso 2010). According to the current KDOQI guidelines for bone metabolism and CKD initial evaluation comprises assessment of calcium and phosphorus metabolism. If needed, the use of phosphate binders is recommended. Further, it is recommended to establish levels of 25(OH)D to prevent or treat insufficiency or deficiency. Levels of 25(OH)D <5 ng/ml or 12nM are indicative of severe vitamin D deficiency, 5-15 ng/ml or 12-37 nM indicate mild deficiency and those of 16-30 ng/ml or 40-75 nM – 25(OH)D insufficiency. Supplementation with vitamin $D_2$ is normally initiated in all three cases (usually stages 3 and 4 CKD), as even in case of 25(OH)D insufficiency it has been shown to reduce the frequency and severity of secondary HPT. In patients with more advanced CKD (stage 5) and in those undergoing dialysis, ergocalciferol or cholecalciferol (i.e. the nutritional replacement) is not recommended as kidneys' ability to generate $1,25(OH)_2D$ is hugely reduced.

In stage 5 of CKD or in dialysis, guidelines recommend therapy with active vitamin D sterol, such as $1,25(OH)_2D$, alfacalcidol, paricalcitol or doxercalciferol. (Noordzij *et al.* 2005) (KDIGO 2009). Treatment with active vitamin D sterols rapidly lowers PTH levels in the serum, improves hyperparathyroidism, bone disease and muskosceletal symptoms. Major side effect of active vitamin D sterols, including $1,25(OH)_2D$ and alfacalcidol is increased intestinal absorption of calcium and

phosphorus, which leads to increased serum levels of those minerals, which eventually may contribute to hypercalcaemia, exacerbates hyperphosphotaemia and reduce bone formation (due to low PTH). In order to reduce or abolish these negative effects, novel analogues of $1,25(OH)_2D$ have been developed (paricalcitol and doxercalciferol – available in USA; maxicalcitol and falecalcitol – available in Asia). Animal studies confirmed that maxicalcitol and paricalcitol are less calceamic and phosphataemic than $1,25(OH)_2D$, whilst effectively suppressing PTH (Nishii *et al.* 1991, Sjoden *et al.* 1984, Slatopolsky *et al.* 1995). Despite some advantages, none of the currently used vitamin D analogues has the ability to selectively target specific tissues, such as kidney or vasculature, instead they induce systemic changes triggered by the endocrine vitamin D system, which in consequence exacerbates the disease progression.

Observational studies demonstrate that both $1,25(OH)_2D$ deficiency and toxicity are common in CKD, narrowing the therapeutic window. In terms of cardiovascular complications, vascular homeostasis is observed within a tight therapeutic range of $1,25(OH)_2D$ treatment, however both vitamin D deficiency and overdose cause vascular damage (*Figure 1.14)* (Querfeld and Mak 2010).

#### *1.8.6.1 Potential Toxic Effects*

Vitamin D toxicity is a result of excessive number of 'free' $1,25(OH)_2D$, unbound to DBP, when there is already an excess of other vitamin D metabolites (Vieth 1990). In rats, $1,25(OH)_2D$ hypercalcaemia is detected, only once the total of all vitamin metabolites exceed approximately 20% of the binding capacity of DBP (Shephard and Deluca 1980).

Previous studies using animal models confirm that the degree of toxicity is correlated to hypercalcaemia, type and concentration of vitamin D metabolite used.

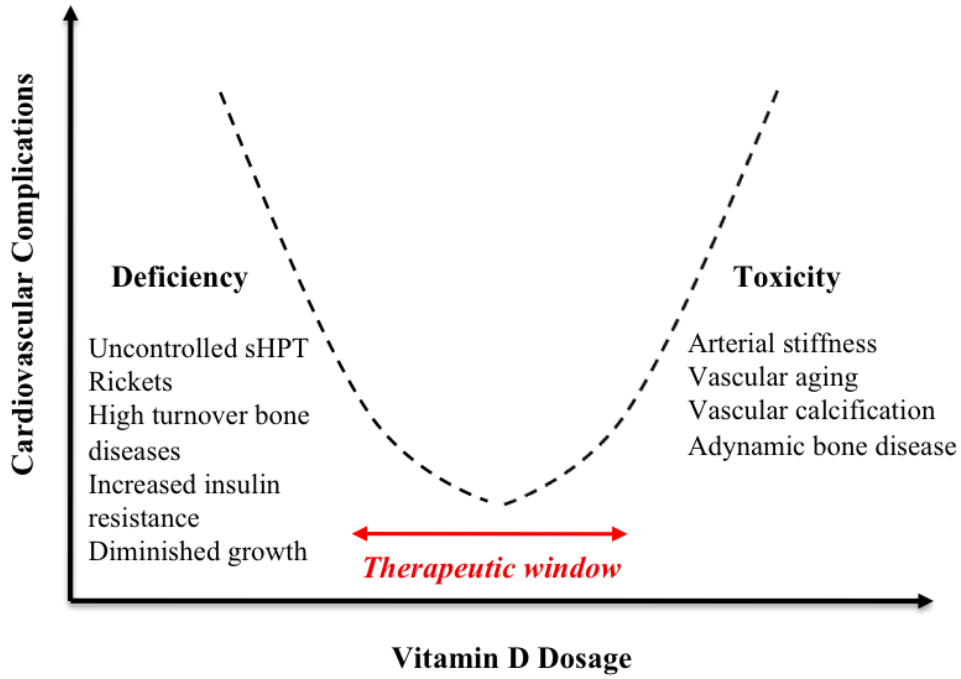


***Figure 1.14: Biphasic U curve representing vitamin D dosage ($1,25(OH)_2D_3$) in relation to cardiovascular complications in children with Chronic Kidney Disease (CKD)***. *sHPT-secondary hyperparathyroidism. Adapted from: (Querfeld and Mak 2010).*

For instance, studies on mice revealed that alfacalcidol is more toxic than $1,25(OH)_2D$, as high doses lead to nephrocalcinosis (Crocker *et al.* 1985). Calcification following $1,25(OH)_2D$ intoxication has been identified in the large number of tissues, particularly kidney, aorta, lung, heart and subcutaneous tissue (Bills 1954, Jones 2008, Vieth 1990).

All, $1,25(OH)_2D$, 25(OH)D and $24,25(OH)_2D$ increase levels of Ca-ATPase, enhance uptake of free calcium and increase cytosolic calcium levels in VSMCs, with $1,25(OH)_2D$ being three times more potent than the other two metabolites (Inoue and Kawashima 1988, Kawashima 1988). This may explain toxicity of $1,25(OH)_2D$, supplementation – coupled with hampered calcium buffering in CKD it may

contribute to accumulation of calcium and phosphate product initiating the process of vascular calcification and influencing arterial tone. Oversuppresion of PTH with active vitamin D can lead to the development of adynamic bone disease (Brandenburg 2008). Also, decreased activity of 1α-OHase mentioned earlier, is not the main obstacle in the treatment of patients with CKD. Another problem is the occurrence of $1,25(OH)_2D$ resistance due to lower VDR numbers, accompanied by toxic composition of uraemic serum interfering with $1,25(OH)_2D$-VDR binding to DNA (Patel *et al.* 1995). Supplementation with higher doses $1,25(OH)_2D$ in this situation may result in hypercalcaemia, hyperphosphataemia, accumulation of high calcium-phosphate product, eventually leading to the development of VC and higher mortality risk.

Some however disagree - it has been postulated that if high levels of 25(OH)D can bind directly to VDR, this may also be triggering a response (Lou *et al.* 2004). In one of his papers DeLuca supports this notion by arguing that $1,25(OH)_2D$ is not responsible for toxicity, and that in fact toxicity is caused by vitamin D or 25(OH)D. The argument was supported by evidence that 1α-OHase null mice experienced identical toxicity to wild type and measurements confirmed that $1,25(OH)_2D_3$ was not synthesized in the -/- mice, excluding $1,25(OH)_2D_3$ as a toxicant (Deluca H. F. *et al.* 2010).

## 1.9 Research Purpose

Our understanding of molecular mechanisms underlying uraemic arteriopathies is still quite basic. VC is a consequence of uremic stress factors and failure of systemic and local protective mechanisms in the artery wall. Calcium and phosphate take a special place in this process, as they can cause VC and are also the main ingredient

for calcium apatite crystals found in VC. Clinical and animal data have suggested an important contributing factor for accelerated VC may be the failure of the vitamin D hormonal system. Also treatment modalities with VDR activators have been associated with improved cardiovascular survival, the mechanisms of action are not well understood. In particular, it is not known, how the endocrine and the vascular vitamin D hormonal system complement each other (*Figure 1.15*). This is particularly important in the context of known harmful effects after treatment with active vitamin D, resulting in increased serum calcium and phosphate. Targeted utilisation of the vascular vitamin D system could help us to make VDR activation more targeted. For this we first need to characterize the human vascular vitamin D hormonal system in health and disease.

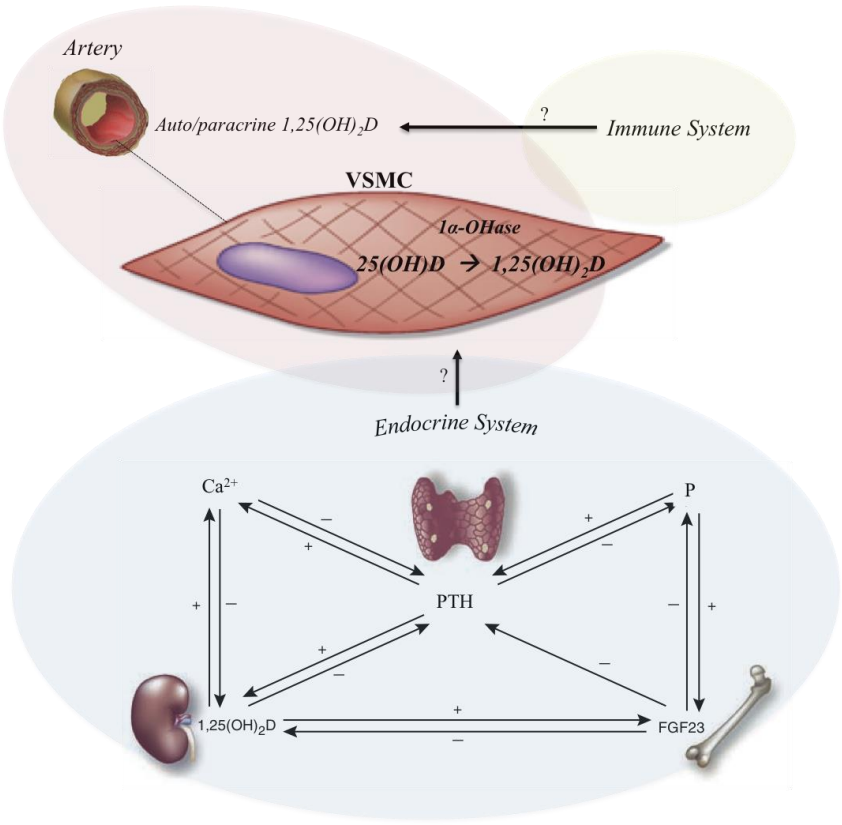


***Figure 1.15*: *Schematic representation of the highly complex interactions between endocrine, para/autocrine and inflammatory control of the vitamin D system in Vascular Smooth Muscle Cells (VSMC).*** *(+) induction, (-) inhibition, (?) effect yet to be determined.*

### 1.9.1 Hypothesis

We postulate that the targeted vascular VDR activation in human, in particular through local metabolic activation, is crucial for vascular health, in particular under stress conditions. We hypothesise that vascular vitamin D system in human is regulated differently to the endocrine vitamin D system and that its regulation is altered in CKD.

***Aims:***

1. *Demonstration of the expression of the vitamin D system in healthy and CKD human artery and in VSMCs.*

2. *Investigation of regulation of the vitamin D hormonal system by 1,25(OH)$_2$D$_3$ in healthy and CKD human artery and in VSMCs.*

3. *Evaluation of regulation of the arterial vitamin D hormonal system by the classical endocrine vitamin D system regulators: calcium, phosphate and inflammatory cytokines (IL-6, IL-17A, TNF-α, INF-γ).*

4. *Evaluation of regulation of the arterial vitamin D hormonal system by the novel endocrine vitamin D system regulators: FGF-23 and Klotho.*

# Chapter 2

# Materials and Methods

## 2.1 Introduction

The procedures described below have all been used within this thesis. Tissue (healthy human arteries, arteries from patients with chronic kidney disease and healthy human kidneys) was obtained with Ethics Committee (for vascular tissue: 05/Q2802/26; for kidney tissue: 10 /H12111/36) and Research & Innovation Department (for vascular tissue and for kidney tissue: DZ077810) approval (Appendix). Informed written consent was obtained from the patient before undergoing a nephrectomy or a kidney transplant at the University Hospital Coventry and Warwickshire NHS Trust, Coventry, UK.

A significant part of my laboratory work included the optimisation of methods to detect and quantify the proteins described in this thesis in human tissue and cells. This extensive development work is explained in this chapter.

## 2.2 Cell and Tissue Culture

### 2.2.1 Principle

Primary cell cultures have been shown to be a valuable tool in cell biology and although they do not reflect the exact physiology of the originating tissue, evidence shows that in certain types of cells, including smooth muscle cells (SMC) the homology to originating tissue is maintained in the early passages. Smooth muscle tissue is found in the *tunica media* of large and small blood vessels and in the walls of hollow organs like the bladder and the uterus. All SMC have the same types of filaments, however, depending on the tissue of origin, they differ significantly in mechanical and physiological properties. Methods for culturing SMCs were published first in 1971 by Campbell, where original Medium 199 was used (Morgan *et al.* 1950). More recently vascular smooth muscle cell (VSMC) culture conditions have been optimised (Dartsch *et al.* 1990) and more advanced basal media are now commercially available (where final supplement concentration after addition to the medium is as follows: 0.5 ml/ml foetal calf serum (FCS), 0.5 ng/ml epidermal growth factor, 2 ng/ml basic fibroblast growth factor, 5 µg/ml insulin).

### 2.2.2 General Methods

All cell and tissue culture procedures were performed under sterile conditions in a Walker Class II Microbiological Safety Cabinet and all media sterilised. Cells in culture were visualised using a Nikon Eclipse TS100 inverted light microscope (Nikon, Surrey, UK). Cultures were maintained in a Heraeus HERAcell 150 incubator (Thermo Fisher Scientific, Leicestershire, UK) at 37°C and humidified

atmosphere containing 5% $CO_2$. All media and culture reagents were pre-warmed to 37°C for at least 45 minutes before use.

#### *2.2.2.1 Primary Human Aortic Smooth Muscle Cell Culture*

Primary SMCs from healthy human aortas (Human Aortic Smooth Muscle Cells, HAoSMCs) were purchased from (TCS Cellworks, Buckingham, UK). They had been isolated from different donors and frozen by the supplier at the end of passage 3 in a medium containing cryoprotectant. On arrival they were store in liquid nitrogen and each vial contained a minimum of 500,000 cells. The approximate doubling time in log phase, as assessed by the supplier, was 27-30 hours and a minimum of 15 doubling times was guaranteed.

The donors included in this study were:

1) 16 year old female – (26274T) – four batches of cells used;

2) 18 year old male (27766T) – two batches of cells used;

3) 43 year old female – (28375) – three batches of cells used.

For culture HAoSMCs, were thawed rapidly at 37°C and transferred to a 75 $cm^2$ flask (BD Falcon) containing 20 ml of pre-warmed Basal HAoSMC medium (TCS Cellworks, Buckingham, UK). All cells were grown routinely in 25$cm^2$ or 75$cm^2$ flasks. Cells were subcultured when they reached 75% confluency using Trypsin-EDTA (0.05%) (Sigma-Aldrich, Dorset, UK). Cells were split 1:2 when 75% confluent. For experimental treatments, cells were trypsinised, pelleted and resuspended accordingly to seed approximately 2,500 cells per $cm^2$ (cells from one 75$cm^2$ flask were split equally between 12 wells of 6-well plates). Cells in individual wells (6-well plate) were counted using the haemocytometer in order to assure

optimally equal distribution across wells. 24h prior to treatments culture medium was replaced with serum-free DMEM/F12 medium containing 0.1% bovine serum albumin (BSA) (Invitrogen, Paisley, UK). Negative controls were included for each individual experiment, where vehicle was added (0.1% ethanol for $1,25(OH)_2D_3$ or $25(OH)D_3$ treatments or 0.1 % BSA for all other treatments).

Long-term primary cell culture (high number of passages) may lead to phenotypic changes in cells. Untreated samples of cells from each passage from different donors were tested for content of α-actin, β-actin, *(Figure 2.1)* and VDR, 1α-OHase and 24-OHase *(Figure 2.2).* As no significant changes were observed between passages 1- and 5, all experiments were performed between those passages.

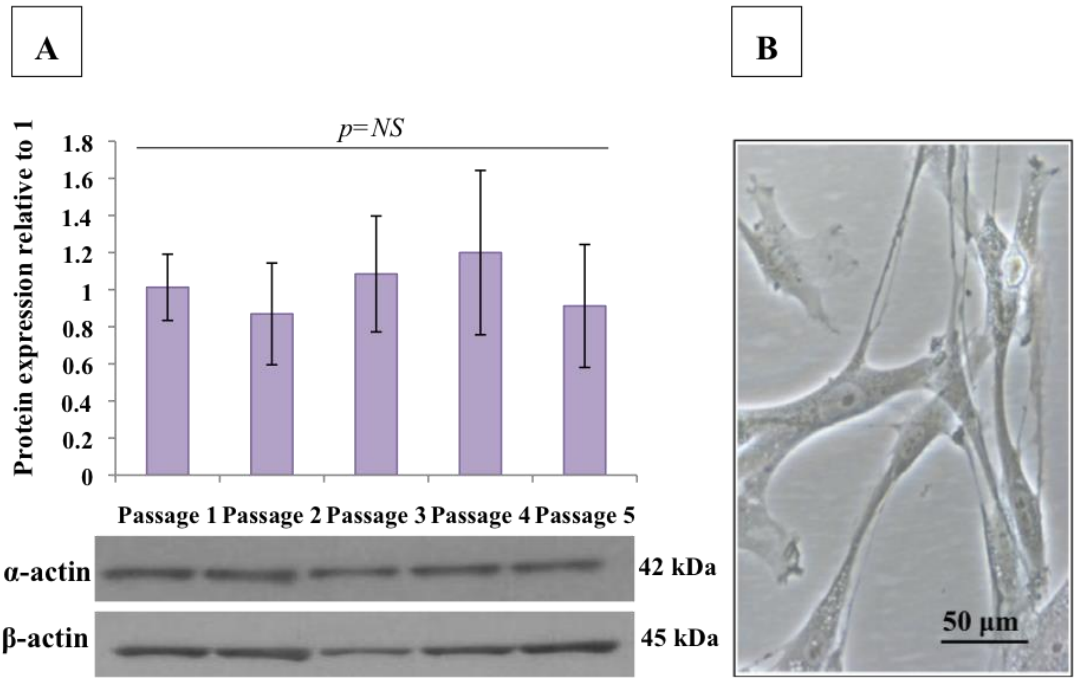


***Figure 2.1 The α-Actin protein expression in HAoSMCs across the consecutive five passages and a microscopic view of a cluster of cells in culture at passage 5.*** *A) Expression of α-actin protein is unchanged between the passages, as demonstrated by Western blot analysis. Protein expression was normalized to levels of β-actin, n=3, p=non-significant (NS), as assessed by one-way ANOVA. B) Individual HAoSMCs in passage 5 of culture, cells appear elongated and spindle like, exhibiting features of a contractile phenotype.*

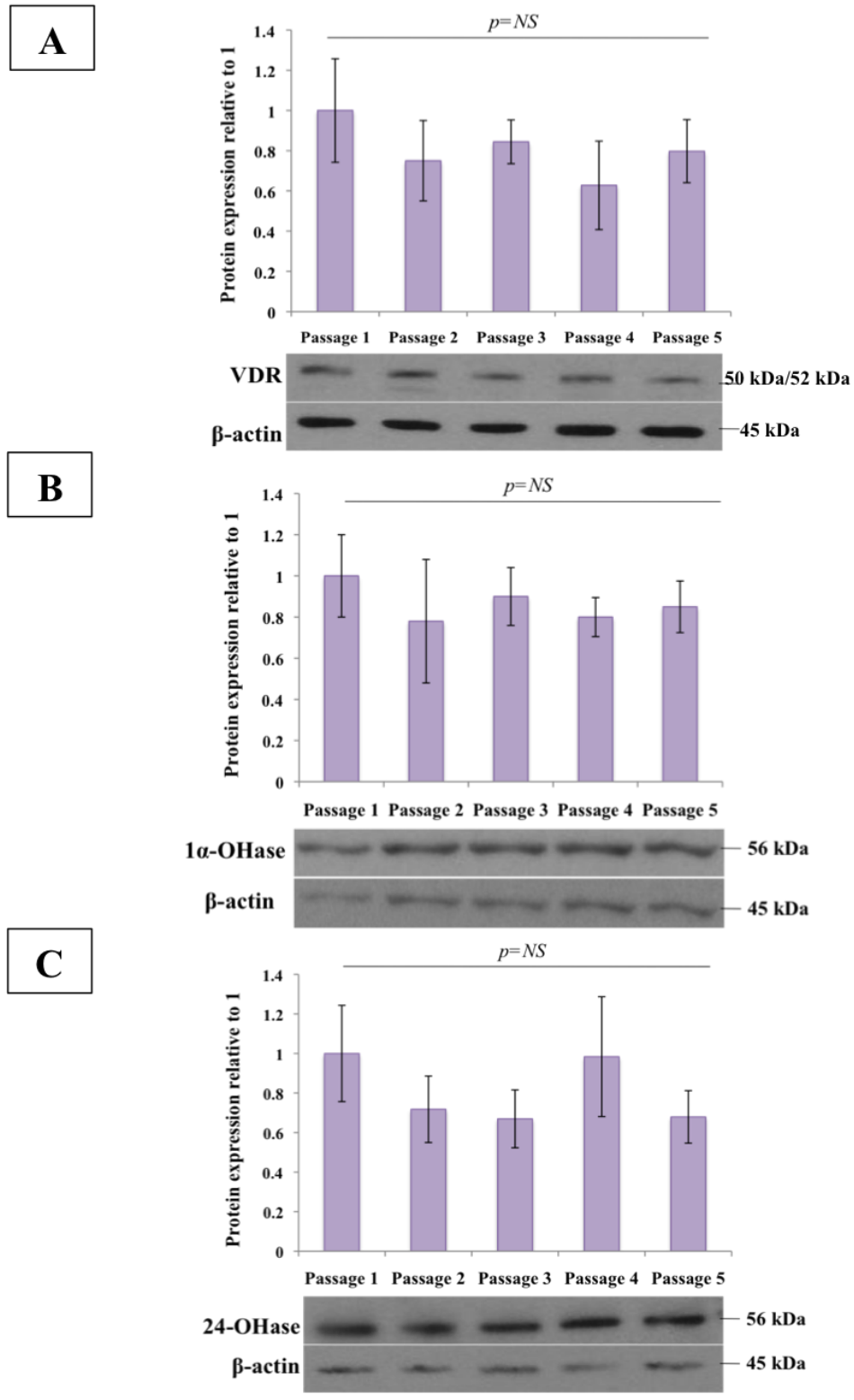


***Figure 2.2: The Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) protein expression across the consecutive five passages, in HAoSMCs****. Protein expression of A) VDR B) 1α-OHase C) 24-OHase is unchanged between five passages, as demonstrated by Western blot analysis. Protein expression was normalized to levels of β-actin, n=3, p=non-significant (NS), as assessed by one-way ANOVA.*

#### *2.2.2.2 Human Proximal Kidney Tubule Cell (HKC-8) Culture*

Human Proximal Kidney Tubule Cells (HKC-8) were kindly provided by Dr Rosemary Bland (University of Warwick, UK) and were used as positive controls in Western blot analyses and VDR antibody validation experiments (isolation of nuclear protein and flow cytometry). Cells were maintained as previously described (Bland *et al.* 1999, Prie *et al.* 1995) in DMEM/F-12 medium supplemented with 5% foetal calf serum (FCS) and 2 mM glutamine. For assessment of mRNA, and protein expression, cells were transferred to defined medium, which consisted of DMEM/F12 containing the following additives: glutamine (2 mM), insulin (5 g/ml), transferrin (5 g/ml), $Na_2SeO_3$ (5 ng/ml), $T_3$ (0.37 nM), epidermal growth factor (2.5 ng/ml), and hydrocortisone (1 nM); Sigma Chemical Co., Poole, UK) for 24 h before adding specific treatment.

### 2.2.3 Cell Counting

Cells grown in 75cm$^2$ flasks were split 1:2 when 75% confluent. For experimental treatments, cells were trypsinised, pelleted and resuspended accordingly to seed approximately 2,500 cells per cm$^2$ (cells from one 75cm $^2$ flask were split equally between 12 wells of 6-well plates). Cells in individual wells (6-well plate) were counted using the haemocytometer in order to assure optimally equal distribution across wells.

### 2.2.4 Arterial Explant Culture

Fresh, surgically removed human renal and epigastric arterial rings (approx. 1-3 mm in length and 1-2 mm in diameter) were maintained as described in 3.1.1. Prior to treatment, arteries were equilibrated and washed for 1h in DMEM/F-12 containing

200 u/ml penicillin and 0.2 mg/ml streptomycin (Sigma-Aldrich, Dorset, UK) *(Figure 2.3).*

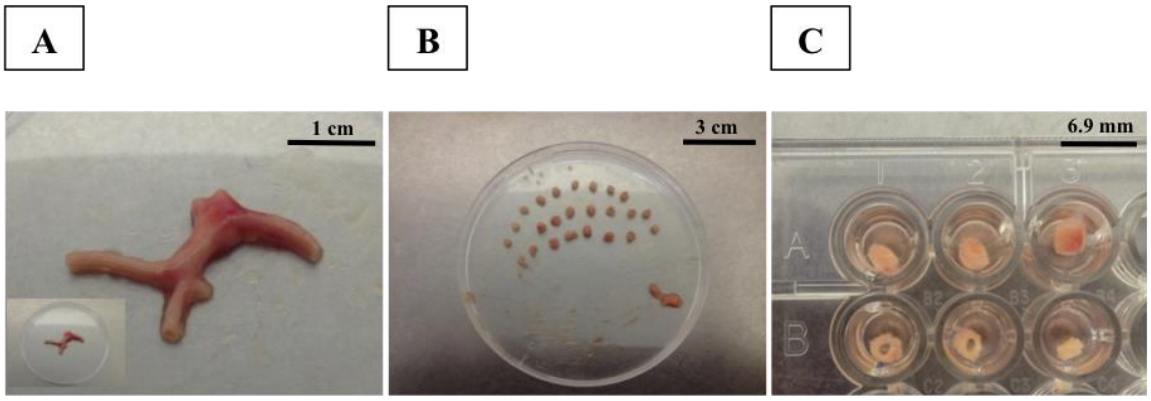


***Figure 2.3: Preparation of artery for organ culture.*** *(A) Artery size relative to Petri dish (9 cm in diameter), with enlarged view of an artery cleared of fat. (B) Artery cut into approximately 0.3cm$^3$ rings. (C) Explants suspended in control or treatment medium (120 µl/well).*

#### *2.2.4.1 Quantification of Cell and Tissue Vitamin D Synthesis: 1,25(OH)$_2$D$_3$ Enzyme Immuno Assay (EIA)*

**HAoSMCs** were grown in 12-well plates and maintained in basal culture medium with 5% FCS or DMEM/F-12 with 10% normal human serum (A mixture of sera collected from healthy individuals, UHCW, UK). Cells, which were grown in basal culture medium with 5% FCS were serum starved overnight by replacing the basal medium with DMEM/F-12. Cells, which were grown in presence of normal human serum, were washed three times with pre-warmed phosphate buffered saline (PBS) before treatment. All cells were treated with DMEM/F-12 with or without 100 nM 25(OH)D$_3$ for 5 h (Sigma-Aldrich, Dorset, UK). Well volume was 400 µl. Where no 25(OH)D$_3$ was added, vehicle (0.1% ethanol) treated 5 h controls were set up. Supernatants were collected and stored at - 80°C. Cells were harvested on ice using

PBS with protease inhibitors (5 mM) (Sigma-Aldrich, Dorset, UK) and three freeze-thaw cycles, as described later in 2.3.2.1

**Arteries from healthy individuals and patients with CKD** were placed in 96-well plates and maintained in DMEM/F-12 with 200 IU/ml penicillin and 0.2 mg/ml streptomycin for one hour. Fresh DMEM/F-12 medium was added with or without 100 nM 25(OH)$D_3$ for 5 h. All supernatants were collected and stored at -80°C. Individual arterial rings were re-suspended in 120 µl of PBS with protease inhibitors and also stored at -80°C.

1,25$(OH)_2D_3$ production (indirect assessment of 1α-OHase activity) will measured by Dr Guerman Molostvov using EIA (IDS, Boldon, UK).

### 2.2.5 Reagents Used in Experimental Treatments

The information on all of the reagents used in experimental treatments is listed in Table 2.1.

***Table 2.1: Reagents used in experimental treatments.***

| ***Reagent*** | ***Company, Catalogue Number*** | ***Treatment Dose/ Dose Range*** |
|---|---|---|
| 1,25 dihydroxyvitamin $D_3$ | Sigma Aldrich, D1530 | 0.1-100 nM |
| 25-hydroxyvitamin $D_3$ | Sigma Aldrich, H4014 | 1-1000 nM |
| Interleukin 6 (IL-6) | PeproTech, 200-06 | 100-200 ng/ml |
| Interleukin 17A (IL-17A) | PeproTech, 200-17 | 200 ng/ml |

| | | |
|---|---|---|
| Tumour Necrosis Factor α (TNF-α) | PromoKine C-63721 | 20 ng/ml, 400 U/ml |
| Interferon γ (INF-γ) | Sigma Aldrich, I3265 | 20 ng/ml, 400 U/ml |
| Calcium Chloride Dihydrate | Sigma Aldrich, 223506 | 1-3 mM |
| β-Glycerol Phosphate Disodium Salt Pentahydrate | Sigma Aldrich,50020 | 1-3 mM |
| Klotho | R&D systems, 5334-KL | 200 pM (0.025 µg/ml) |
| Heparin Sodium | CP Pharmaceuticals Ltd, | 10 µg/ml |
| Fibroblast Growth Factor 23 (FGF-23) | Pepro-tech, 100-52 | 5-100 ng/ml |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma Aldrich, P1585 | 30 µM |
| PD98059 (MEK1 Inhibitor) | Calbiochem, 513000 | 60 µM |
| Coctail of Protease Inhibitors | Sigma Aldrich, P8340 | 5 mM |

## 2.3 Western Blot Analysis

### 2.3.1 Principle

Western blotting is a sensitive technique used to identify and semi-quantify proteins in complex protein mixtures from cell or tissue samples. After separation of proteins by gel electrophoresis (Burnette 1981, Towbin *et al.* 1979) they are transferred to a suitable membrane (e.g. polyvinylidene fluoride- PVDF or nitrocellulose). Due to a spatial resolution, this method provides molecular weight information on individual proteins. Once proteins have been transferred onto a membrane, they can be stained

for visualisation and identified by N-terminal sequencing, mass spectrometry or immunodetection.

There are many types of electrophoresis, such as negative gel electrophoresis, electrofocusing gel electrophoresis, etc., however the one used in this study was sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE). SDS-PAGE is carried out in the presence of sodium dodecyl sulfate as the primary solubilising and charge-conferring agent. SDS binds to linearised proteins, which results in even distribution of charge per unit mass, ultimately allowing separation by molecular weight during electrophoresis. Therefore SDS-PAGE is used to separate proteins accordingly to their electrophoretic mobility, which is a function of the charge of the polypeptide chain and its length. The mobility of the ions is pH-dependent and the higher the pH, the faster the moving boundary (the more the system is able to separate low molecular mass proteins, at the expense of the separation of high molecular mass proteins). Conversely, the lower the pH, the more space is allocated to separate high molecular mass proteins, with the low molecular mass ones co-migrating with the ion boundary. The pH in gels is maintained by various buffers systems, with Tris-HCl being the most widely used. The first SDS-PAGE was described by Laemmli over four decades ago (Laemmli 1970). Ever since then a variety of 2-D acrylamide gels have been developed, utilizing new polymers, such as Duracyl or changing the monomers from acrylamide and bis-acrylamide to Bis-acryloyl piperazine and acryloyl morpholine (Artoni *et al.* 1984, Rabilloud 2010). A number of permutations in protocols and techniques exist, being constantly perfected, each optimised to separation and ultimately – detection of the proteins of interest.

## 2.3.2 Preparation of Protein for Western Blot Analysis

### *2.3.2.1 Protein Isolation from Cells*

Three protein harvest buffers were tested: RIPA, modified-RIPA (Halford and Russell 2009) and PBS, all three with protease inhibitors, in order to subsequently achieve the optimal VDR, 1α-OHase and 24-OHase protein detection. Cells were placed on ice and washed three times with chilled PBS and any leftover PBS was carefully aspirated and discarded. Cells were solubilised in PBS with protease inhibitors and triple freeze-thawed by (25°C vs. -80°C) to facilitate the rupture of cell membranes. All lysates were centrifuged at 4000 g for 10 minutes to pellet the cell debris and the supernatant was collected for subsequent protein concentration measurement. Lysates were aliquoted and stored at -80°C.

Test Buffers' Constituents (all reagents were purchased from Sigma-Aldrich, Dorset, UK):

- **RIPA buffer** (Millipore, Nottingham, UK), constituents:

  - 1% Nonidet P-40 (surfactant, non-ionic detergent to extract proteins)
  - 0.25% Sodium Dodecyl Sulphate (surfactant, ionic detergent to extract proteins)
  - 50 mM Tris Hydrochloride (buffering agent prevents protein denaturation)
  - 150 mM Sodium Chloride (salt, prevents non-specific protein aggregation)
  - 1 mM Ethylene Glycol Tetraacetic Acid- EGTA (chelating agent)
  - 0.5 M Cocktail of Protease Inhibitors
  - 0.5 M Phosphatase Inhibitors
  - pH 7.4

- **Modified RIPA buffer** (self-made), constituents:

  - 1% Nonidet P-40
  - 0.1% Sodium Dodecyl Sulphate

- 0.5% Sodium Deoxycholate (surfactant, ionic detergent to extract proteins)
- 50 mM Tris-Hydrochloride
- 150 mM Sodium Chloride
- 0.5 M Cocktail of Protease Inhibitors
- 0.5 M Phosphatase Inhibitors
- pH 8.0

- **PBS buffer (self-made),** constituents:

  - Phosphate Buffered Saline (Potassium Chloride 2.7 mM, Sodium Chloride 137 mM, Sodium Phosphate Dihadrate 10 mM, Potassium Phosphate 2mM)
  - 0.5 M cocktail of protease inhibitors
  - pH 7.4

PBS with protease inhibitors optimally preserved protein and therefore was used routinely in 1α-OHase, 24-OHase and VDR experimental work (*Figure 2.4*).

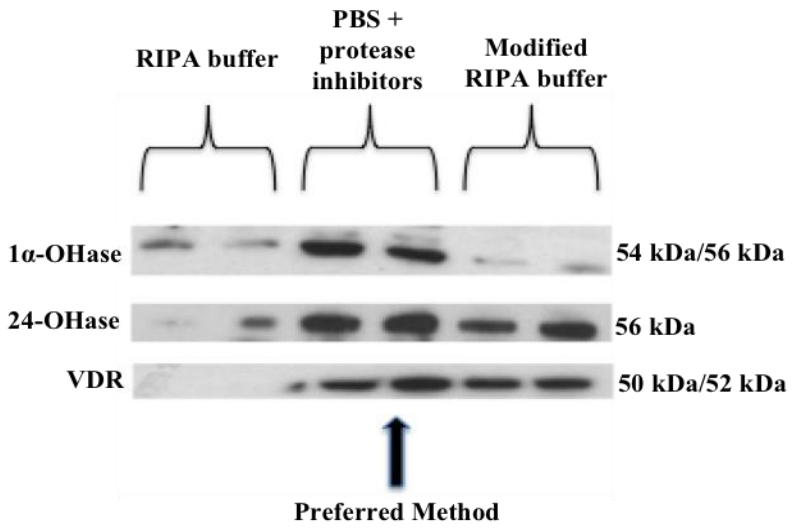


***Figure 2.4: Selection of the optimal protein preparation method****. Expression of 1α-Hydroxylase (1α-OHase), 24-Hydroxylase (24-OHase) and Vitamin D Receptor (VDR) protein isolated from HAoSMCs in three different buffers (RIPA, PBS and modified RIPA, all with cocktail of protease inhibitors). Three separate cell lysates were prepared. Proteins were separated by 10% SDS-PAGE and Western blotted with 1α-OHase, 24-OHase or VDR antibodies. PBS with protease inhibitors appeared to be the optimal buffer, with visibly higher expression of protein, compared to other two buffers. 10 μg protein per lane, loaded in duplicate.*

#### *2.3.2.1.1 Protein Isolation from Cells for Subsequent β-Actin, α-Actin, Klotho, FGFR1, FGFR3, RUNX-2, ALP, Sclerostin Protein Detection*

The protocol was the same as described in 2.3.2.1 except that RIPA buffer containing protease inhibitors (5 mM) was used.

### *2.3.2.2 Protein Isolation from Tissue*

Approximately 0.3cm$^3$ of the required tissue was ground in liquid nitrogen in 0.2 ml of PBS containing a complete cocktail of protease inhibitors (5 mM) for the characterisation of (VDR, 1α-OHase and 24-OHase) or RIPA buffer with protease inhibitors (5 mM) for β-actin, α-actin, Klotho, FGFR1, FGFR3, RUNX-2, ALP and sclerostin protein using a homogeniser. The lysates were then freeze-thawed (three times, as mentioned previously for VDR, 1α-OHase and 24-OHase only) and centrifuged at 300 g for 10 minutes at 4°C. The supernatant was collected, aliquoted and stored at -80°C.

## 2.3.3 Measurement of Protein Concentration

### *2.3.3.1 Principle*

The total soluble protein concentration of cell extracts or tissue homogenates was measured using Bio-Rad Protein Determination Kit (Bio-Rad Laboratories, Hertfordshire, UK) with a standard curve constructed from BSA (0-1.4 mg/ml in either PBS or RIPA buffer). Due to a constant extinction coefficient over a 10-fold concentration range, a dye-albumin complex within the linear range could be obtained (Bradford 1976).

#### *2.3.3.2 Method*

The reaction was performed in 96-well plate where the protein standard, or the samples of cell/tissue protein lysates (5µl/well) were mixed 1:5 with previously prepared alkaline copper tartrate solution (4:1, vol./vol. concentrate and buffer). Next, 200 µl of folin reagent was added to each well, placed on a plate shaker for 15 seconds and incubated at 25°C for 10 minutes. Reduction of folin by the copper-treated protein (tyrosine and tryptophan residues, but also cystine, cysteine, and histidine) produced a blue color. Absorbance at 650 nm was measured using a MultiScan Ascent plate reader (Thermo Electron Corporation), with background values determined by the working dye solution with no protein.

### 2.3.4 Separation of Protein by Electrophoresis and Protein Transfer

Appropriate volumes of protein lysates, containing 10 µg of protein were mixed with Laemmli Sample Buffer containing 5% β-mercapthoethanol (Bio-Rad Laboratories, Hertfordshire, UK) in a ratio 1:1. The sample dilution was adjusted with water so that an equal volume was loaded per lane. Samples were denatured by heating for 5 minutes at 95°C, briefly centrifuged and loaded. Gels were run in the electrophoresis tanks with 500 ml of electrode buffer. Transfer was achieved using a wet or semi-dry system (for details see sections: 2.3.4.1.2-VDR, 2.3.4.1.4-1α-OHase, 2.3.4.1.6- 24-OHase, 2.3.4.2- all other proteins).

#### *2.3.4.1.1 Optimisation of Detection and Quantification of the VDR Protein in Cells and Tissues*

Pre-cast Nu-PAGE 10% Bis-Tris gel (Invitrogen, Paisley, UK) and pre-cast RunBlue 10% Bis-Tris gel (Expedeon, Harston, UK), both with buffers recommended by manufacturers, were tested.

Results showed that the optimal protein separation was achieved using the pre-cast Nu-PAGE 10% Bis-Tris gel (*Figure 2.5*).

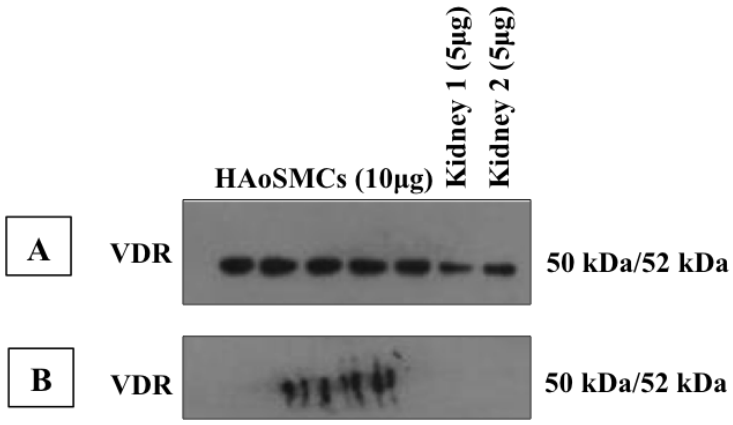


***Figure 2.5 The comparison of the Vitamin D Receptor (VDR) protein separation and detection by two commercially available pre-cast gels.*** *A) Nu-Page 10% Bis-Tris Gel, Invitrogen and B) RunBlue 10% Bis-Tris gel, Expedeon. Transfer was achieved using nitrocellulose membrane and a wet system (100 V for 1 h). Membranes were blocked in Animal Serum Free Block (ASFB) for 1h at 25°C. Membranes were probed with primary rabbit polyclonal VDR C-20 antibody (1:500) in ASFB overnight at 4°C, washed with PBS-T (0.1%) and probed with secondary (horse-raddish-peroxidase-conjugated) antibody (1:4,000) in ASFB.*

Further optimisation of the method was required for the comparison of 11 or more artery samples on one gel (pre-cast gels were not appropriate, due to a too small number of wells). Consequently, four different methods were compared: A) self-made 10% polyacrylamide gel and a nitrocellulose membrane, B) pre-cast Nu-PAGE 10% Bis-Tris gel and nitrocellulose membrane, C) self-made 10% polyacrylamide gel and a PVDF membrane, D) pre-cast 10% Bis-Tris gel and a PVDF membrane.

Transfer was performed at 100 mA for 2 h, with the use of GeneFlow transfer buffer. Method C provided the optimal result (*Figure 2.6*).

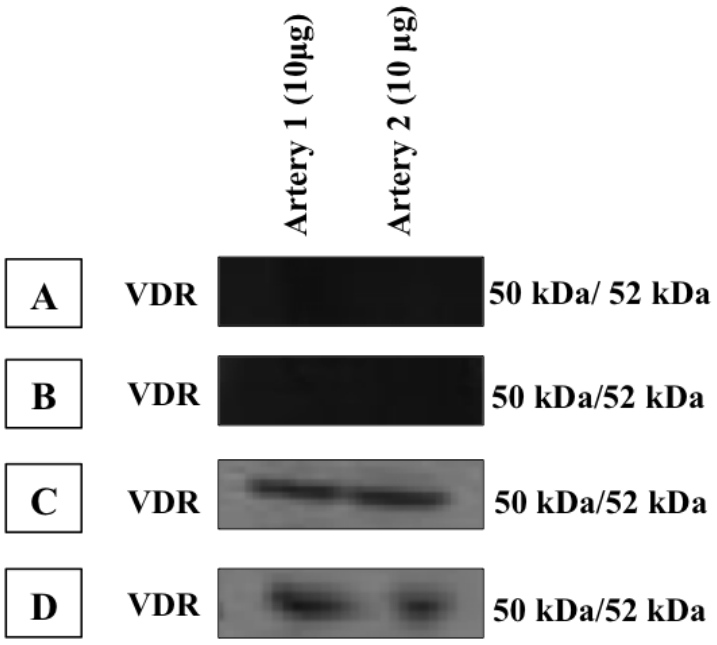


***Figure 2.6: Transfer optimisation for the Vitamin D Receptor (VDR) protein isolated from human artery.*** *A) Self-made 10% polyacrylamide gel and a nitrocellulose membrane B) Pre-cast Nu-PAGE 10% Bis-Tris gel and nitrocellulose membrane C) Self-made 10% polyacrylamide gel and a PVDF membrane D) Pre-cast 10% Nu-Page Bis-Tris gel and a PVDF membrane. Electrophoresis was performed at 140 V for 70 minutes, semi-dry transfer at 100 mA for 2 h, blocking in Animal Serum Free Blocker (ASFB) for 1 h at 25°C, primary antibody (VDR-C20) 1:500 in ASFB at 4°C overnight, secondary antibody, HRP-conjugated anti-rabbit 1:4,000 in ASFB.*

It was evident that after longer exposure (15 seconds-1 minute) a second band appeared in HAoSMCs and HKC-8 samples (*Figure 2.7*).

In order to check whether the antibody was binding specifically to VDR, nuclear protein from HAoSMCs and HKC-8 (control) were isolated and probed for VDR.

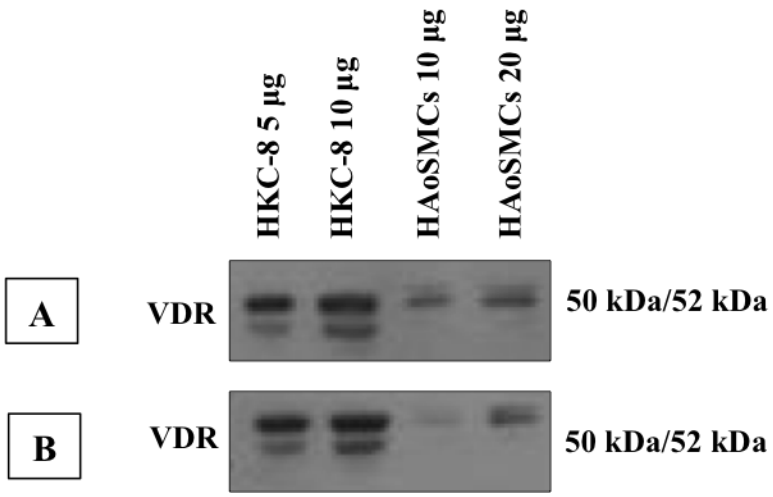


***Figure 2.7: The Vitamin D Receptor (VDR) protein expression in Human Proximal Tubule Cells (HKC-8) and in Human Aortic Smooth Muscle Cells (HAoSMCs) – the optimised method.*** *Double bands (approx. 50 and 52 kDa) were detectable in both cell types, although the 50 kDa band was hardly visible in HAoSMCs at given exposure. There appeared to be an additional band of approx. 53 kDa present in HAoSMCs. Electrophoresis was performed at 140 V for 70 minutes, semi-dry transfer at 100 mA for 2 h, blocking in Animal Serum Free Block (ASFB) for 1 h at 25°C, primary antibody (VDR-C20) 1:500 in ASFB at 4°C overnight, secondary antibody, HRP-conjugated anti-rabbit; A) 1:5,000 in ASFB or B) 1:4,000 in ASFB.*

#### *2.3.4.1.1.1 Preparation of Nuclear Proteins for VDR Western Blot Analysis*

Nuclear proteins were extracted using an adaptation of the method of Samuels and Tsai (Samuels *et al.* 1974). Cells were lysed in a sucrose/Tris/MgCl buffer containing Triton X-100 and the nuclear proteins were released from the nuclei by incubation in a KCl lysis buffer. This can only be achieved in presence of protease inhibitor, without which a loss of as much as 95% of nuclear protein within 4 minutes can occur (Paine *et al.* 1983).

Cells from each 75 $cm^3$ flask were washed with PBS and scraped as normal and centrifuged at 4000 g for 5 minutes to pellet. Supernatant was discarded and cells were washed with PBS and centrifugation step was repeated. Pellet was resuspended in 4 ml of STM buffer (0.25 M sucrose, 20 mM Tris, 1.1 mM $MgCl_2$ with 0.5 mM

protease inhibitors) and centrifuged at 900 g for 10 minutes at 4°C, to separate whole cells. Pellets were re-suspended in STM buffer with protease inhibitors and 0.5% Triton to rupture cell membranes and left on ice for 10 minutes. Subsequently, the suspension was centrifuged as previously to separate the nuclei. Pelleted nuclear fraction was then re-suspended in STM buffer with protease inhibitors and 0.5% Triton, left on ice for 10 minutes and centrifuged as previously. 400 µl of Lysis Mixture (STM with 0.5 mM PIs, 5mM DL-dithiothreitol (DTT), 4M KCl, 20% vol./vol. glycerol) was added and samples were left agitating for 15 minutes at 4°C. Following final centrifugation at 4000 g for 15 minutes at 4°C, supernatant containing nuclear proteins was collected, aliquoted and stored at -80°C.

Results showed that both the whole cell lysates and the nuclear extracts contained the VDR protein *(Figure 2.8).*

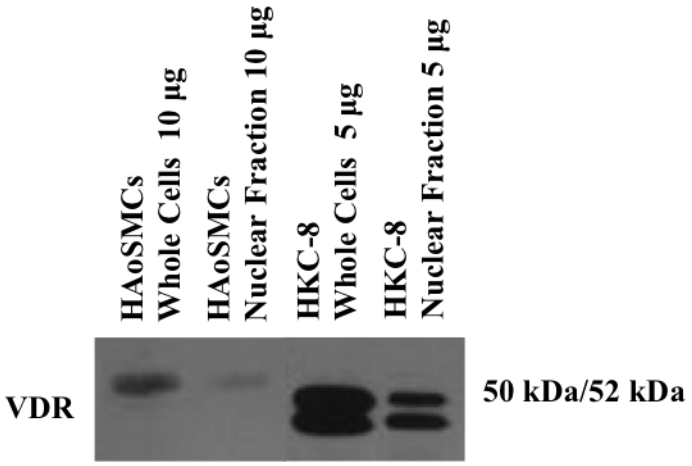


***Figure 2.8: The Vitamin D Receptor (VDR) protein is expressed in both, whole HAoSMCs and the isolated nuclear fraction****. Doublet of bands of about 48 and 50 kDa was detectable in HKC-8 samples, 50 and 52 kDa in HAoSMCs. Electrophoresis was performed at 140 V for 70 minutes, semi-dry transfer at 100 mA for 2 h, blocking in for 1 h at 25°C, primary antibody (VDR-C20 1:500 in ASFB at 4°C overnight, secondary antibody, HRP-conjugated anti-rabbit 1:4,000 in ASFB.*

#### *2.3.4.1.2 FINAL METHOD USED for the VDR Protein Detection and Quantification in Cells and Tissues*

Denatured samples were loaded into NuPAGE 10% Bis-Tris pre-cast polyacrylamide gels using the mini-cell SDS-PAGE system (Invitrogen, Paisley, UK). The running buffer was prepared for denaturing electrophoresis using the recommended protocol provided with NuPAGE MOPS SDS running buffer (Invitrogen, Paisley, UK). 500 μl of antioxidant (Invitrogen, Paisley, UK) was added to the running buffer and electrophoresis was performed at 140V for 70 minutes at room temperature - until adequate separation of the molecular marker protein was achieved (Fermentas, York, UK).

Following electrophoresis, proteins were transferred onto nitrocellulose membrane. Transfer was achieved using wet-blot transfer system (Bio-Rad Laboratories, Hertfordshire, UK), after 1 hour at 100 V. Standard transfer buffer containing 00.2 M Tris, 0.19 M glycine, 20% (vol./vol.) methanol, pH 8.3 (Geneflow, Stafford, UK) was used.

Membranes were blocked (1 h; 25 °C) in Animal Serum Free Block (ASFB, Vector Labs, Peterborough, UK) and rinsed in PBS. Membranes were incubated overnight at 4 °C with the primary rabbit VDR C-20 antibody diluted 1:500 (Santa Cruz Biotechnology, Santa Cruz, USA) in ASFB. After three 10-min washes in PBS-T (0.1%), membranes were incubated with the secondary antibody (horseradish peroxidase conjugated, Vector Labs, Peterborough, UK) diluted 1:4,000 in ASFB for 60 minutes at 25 °C and washed for three 10-minute periods in PBS-T (0.1%). Proteins were detected by enhanced chemiluminescent assay (ECL, Amersham, Buckingham, UK) after exposure of filters to X-ray film for 30 seconds – 3 minutes).

Autoradiographs were quantified by densitometry, using Image J software. Signal emitted from the peroxidase-conjugated secondary antibodies created darker bands on the X-ray film. Optical Density (O.D.) reflects the amount and intensity of pixels in the scanned image and can be measured by calculating the surface area under a computer-generated curve (proportional to the amount of target protein).

#### *2.3.4.1.3 Optimisation for the Characterisation and Quantification of 1α-OHase Protein in Cells and Tissues*

The specific method for Western blot analysis for 1α-OHase protein has been described previously (Bland *et al.* 1999).

This and three other methods were compared in HAoSMCs. All methods were based on the original published method, except: Method A: blocking in I-Block (Invitrogen, Paisley, UK), Method B: blocking in ASFB, Method C: blocking in non-fat milk powder 5% (wt/vol.), Method D: blocking in non-fat milk powder 20% (wt/vol.). The method D as described by Bland *et al.* provided the optimal result and was used for analyses of 1α-OHase protein isolated from HAoSMCs and tissues, (2°Ab. 1:20,000) (*Figure 2.9)*.

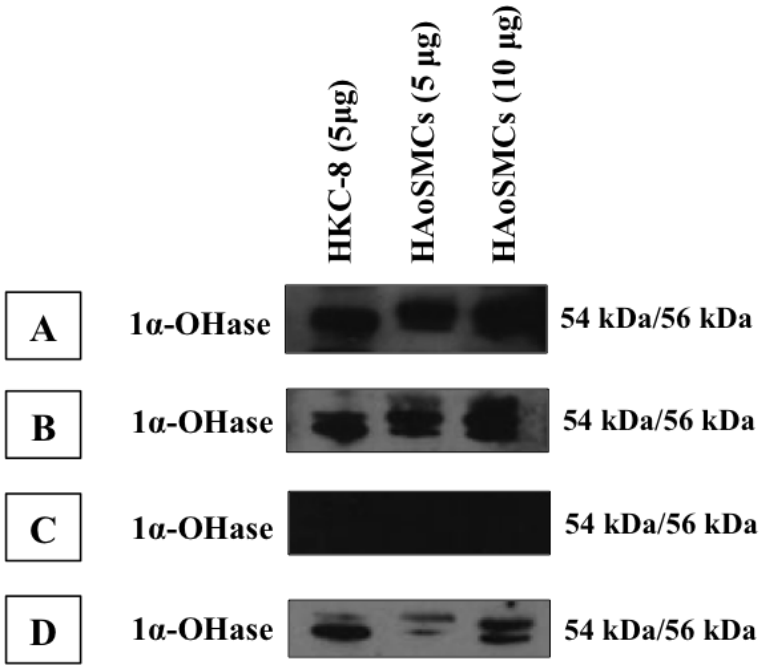


***Figure 2.9: The optimisation of blocking methods for detection of 1α-Hydroxylase (1α-OHase) protein.*** *A) I-block B) Animal Serum Free Block (ASFB) C) non-fat milk powder 5% (wt/vol.) D) non-fat milk powder 20% (wt/vol.). Proteins were transferred from self-made 10% polyacrylamide gels onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in I-block or ASFB or 5% or 20% (wt/vol.) non-fat milk powder for 1h at 25°C. Membranes were incubated overnight at 4 °C with the primary mouse 1α-OHase antibody diluted 1:500 in buffers (0.05% PBS-T). Secondary antibody (horse radish peroxidase-conjugated) was applied, diluted 1:20,000 in PBS-T (0.1%). Membranes were washed with 0.1% PBS-T between each step.*

In both HAoSMCs cells and kidney, a double band of approximately 54 and 56 kDa, was detectable. In tissues, the appearance of the same doublet was even more prominent with additional band of approximately 70 kDa, which was absent from the HAoSMC protein lysate preparation. The specificity of mouse 1α-OHase antibody (The Binding Site, Birmingham, UK) was therefore assessed by antibody pre-incubation with a 100-fold excess of immunising peptide.

**Method Development for the 1α-OHase Antibody Neutralisation**

0.5 µg of custom made 1α-OHase peptide, with the following amino acid sequence: RHVELREGEAAMRNQGKPEEDMPS (AltaBioscience, Birmingham, UK) was dissolved in 1ml of sterile PBS overnight. 20 µl of dissolved peptide were mixed with 8 µl of anti-1α-OHase antibody (100-fold excess of peptide to ensure sufficient antibody neutralisation), overnight at 4°C in 2ml of PBS and then added to the same volume of PBS-T (0.1%). This resulted in neutralisation of the smaller set of bands (54 and 56 kDa) in normal human arteries, HAoSMCs and kidney. The neutralisation was not as prominent in kidney, due to significantly higher level of 1α-OHase protein expression *(Figure 2.10).*

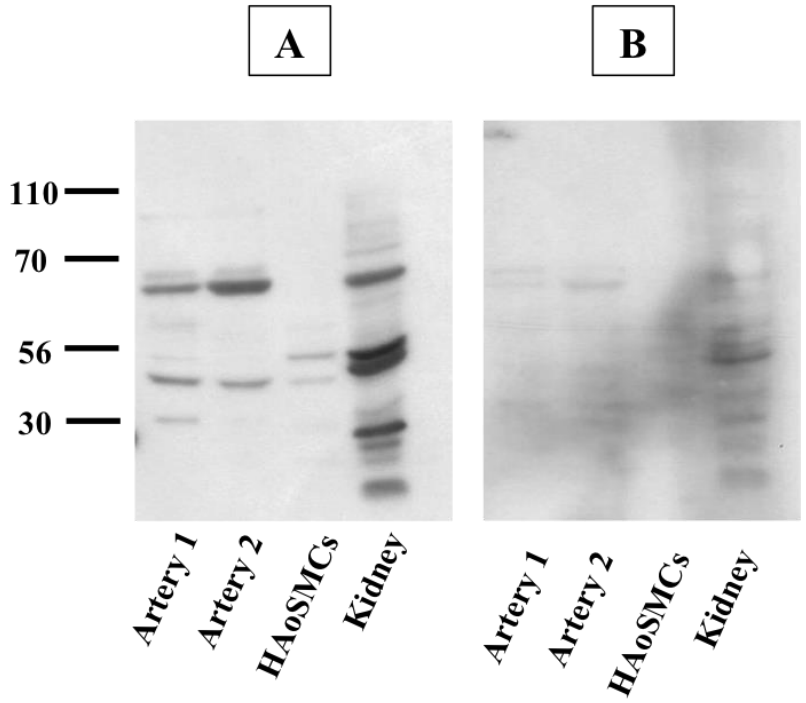


***Figure 2.10: Neutralisation of 1α-Hydroxylase (1α-OHase) protein.*** *Expression of 1α-OHase in human artery, HAoSMCs and kidney in presence and absence of antibody-neutralising peptide. Western blot analysis of protein preparation (10 µg) from normal renal arteries (artery 1 and artery 2), HAoSMCs and a normal kidney (A) in the presence or (B) absence of 100-fold excess of immunising peptide. 1α-OHase molecular weight: 56 kDa.*

#### *2.3.4.1.4 FINAL METHOD USED for Detection and Quantification of 1α-OHase Protein in Cells and Tissues*

SDS-polyacrylaminde gels for 1α-OHase were self-made and the constituents are listed below.

*Constituents for the resolving and stacking gels:*

**10% resolving gel:**

- 4 ml 30% (vol./vol.) Acrylamide 37.5:1 (National Diagnostics, Hessle, UK)
- 3ml of 1.5 M Tris-Glycine Buffer pH 8.8 (Bio-Rad, Herefordshire, UK)
- 120 µl 10% (wt/vol.) Sodium Dodecyl Sulphate (Sigma-Aldrich, Dorset, UK)
- 4.84 ml distilled $H_2O$
- 40 µl 10% (wt/vol.) Ammonium Persulphate (Sigma-Aldrich, Dorset, UK)
- 10 µl TEMED (Bio-Rad, Herefordshire, UK)

**4.5% stacking gel:**

- 4 ml 40% (vol./vol.) Acrylamide 29:1 (Bio-Rad, Herefordshire, UK)
- 2.5 ml 0.5 M Tris-Glycine Buffer pH 8.8 (Bio-Rad, Herefordshire, UK)
- 100 µl 10% (wt/vol.) Sodium Dodecyl Sulphate (Sigma-Aldrich, Dorset, UK)
- 6 ml distilled $H_2O$
- 100 µl 10% (wt/vol.) Ammonium Persulphate (Sigma-Aldrich, Dorset, UK)
- 15 µl TEMED (Bio-Rad, Herefordshire, UK)

The ingredients of the resolving gel were mixed and the gel solution was poured into the gel mould to the required height. The surface was gently overlaid with water and the gel was left to polymerise for about 1 hour. When the resolving gel had polymerised the water was removed and the stacking gel was mixed and poured over the resolving gel. The combs were inserted and the gel was left to polymerise for 1 hour. Just before the use the combs were removed and the wells were rinsed with water.

The samples were prepared for loading as described in 2.3.4. Denatured samples were loaded into self-made 10% SDS-polyacrylamide gel. The electrode buffer was 0.02 M Tris with 0.19 M glycine and 3.4 M SDS. Electrophoresis was performed at 200 V for 40 minutes at 4°C, until adequate separation of the molecular weight marker protein (Fermentas, York, UK) was achieved.

Following electrophoresis, proteins were transferred onto PVDF membrane – Immobilon-P (Millipore, Dundee, UK), which was previously briefly immersed in methanol, to wet the membrane (Sigma-Aldrich, Dorset, UK), then water for 1-2 minutes, to elute the methanol, and equilibrated in transfer buffer for 20 minutes. Transfer was achieved using wet-blot transfer system (Bio-Rad, Hertfordshire, UK), after 1 hour at 100 V. Transfer buffer was of 0.06 M Tris, 0.48 M Glycine and 20% (vol./vol.) methanol. Membranes were blocked (1 h; 25 °C) in PBS-T (0.1% containing 20% (wt/vol.) non-fat milk powder (Marvel, Premier Brands, Stafford, UK) and rinsed twice in PBS, followed by an additional wash in PBS-T for 15 minutes. Membranes were incubated overnight at 4 °C with the primary mouse 1α-OHase antibody diluted 1:500 (The Binding Site, Birmingham, UK) in PBS-T (0.05%). After three 10-min washes in PBS-T (0.1%), membranes were incubated with the anti-mouse secondary antibody (horseradish peroxidase conjugated, The Binding Site) diluted 1:20,000 in PBS-T (0.1%) for 60 minutes at 25 °C and washed for three 10-minute periods in PBS-T (0.1%).

Proteins were detected by the ECL after the exposure of filters to X-ray film for 30 seconds – 3 minutes). Autoradiographs were quantified by densitometry, using Image J software as described for VDR.

#### *2.3.4.1.5 Optimisation for Characterisation and Quantification of 24-OHase Protein in Cells and Tissues*

Two different 24-OHase antibodies (custom made sheep polyclonal antibody – The Binding Site, Birmingham, UK) and commercially available mouse monoclonal antibody, clone 1F8 – Sigma-Aldrich, Dorset, UK) were tested using HAoSMCs protein obtained by three different harvest methods (as described in 2.3.2.1.1). Two different blocking conditions were used (non-fat milk powder 5% or 20% (wt/vol.) at room temperature, for 1 hour. Application of 1F8 antibody, to the method described for 1α-OHase Western analysis (see 2.3.4.1.4) resulted in clear blots and production of clear specific bands of 56 kDa (*Figure 2.12*).

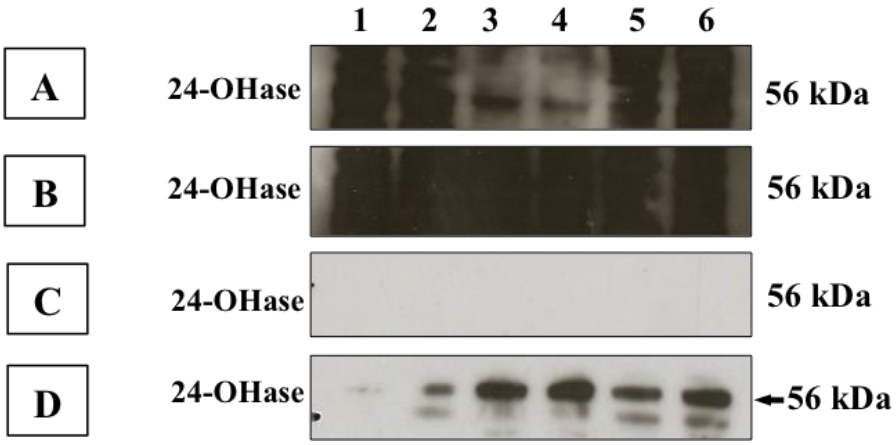


***Figure 2.11: The optimisation of 24-Hydroxylase (24-OHase) protein detection in Human Aortic Smooth Muscle Cells (HAoSMCs) using two different antibodies.*** *(A,B): The Binding Site (C,D): Sigma-Aldrich (A,C): Blocking in 5% (wt/vol.) non-fat milk powder 1°Ab. 1:500 in PBST 0.05%, 2°Ab. peroxidase conjugated 1:5,000 in 5% (wt/vol.) non-fat milk powder. (B,D): Blocking in 20% wt/vol.) non-fat milk powder, 1°Ab. 1:500 in PBST 0.05%, 2°Ab. peroxidase conjugated 1:5,000 in 5% (wt/vol.) non-fat milk powder. Lanes: 1,2: HAoSMCs 10 µg/lane harvested in RIPA with protease inhibitors, 3,4: HAoSMCs 10 µg/lane harvested in PBS with protease inhibitors, 5,6: HAoSMCs 10 µg/lane harvested in Modified RIPA with protease inhibitors.*

#### *2.3.4.1.6 FINAL METHOD USED for Detection and Quantification of 24-OHase Protein in Cells and Artery*

The protocol was identical to one described earlier for 1α-OHase (see 2.3.4.1.4), except the membranes were incubated overnight at 4 °C with primary mouse 24-OHase antibody diluted 1:500 (Sigma-Aldrich, Dorset, UK) in PBS-T (0.05%). After three 10-min washes in PBS-T, membranes were incubated with the secondary antibody (horseradish peroxidase conjugated) (Vector Labs, Peterborough, UK) diluted 1:5,000 in 5% (wt/vol.) non-fat milk powder (Marvel, Premier Brands, Stafford, UK) in PBS-T (0.1%) for 60 minutes at 25°C and washed for three 10-minute periods in PBS-T. Proteins were detected by ECL Filters were exposed to X-ray film for 2–40 minutes). Autoradiographs were quantified by densitometry, as described for VDR.

### *2.3.4.2 Detection and Quantification of α-Actin, β-Actin, Klotho, FGFR1, FGFR3, RUNX-2, ALP, Sclerostin, ERK1/2 and P-ERK Protein*

Western analyses of other proteins (α-actin, β-actin, Klotho, FGFR1, FGFR3, RUNX-2, ALP, sclerostin, ERK1/2 and P-ERK) were performed using 10% self-made gels (as described in 2.3.4.1.4) or (8% for Klotho, for ingredients see below). The electrode buffer was identical to the one used for 1α-OHase and 24-OHase (2.3.4.1.4).

*Constituents for the resolving gel:*

**8% resolving gel:**

- 6 ml 40% (vol./vol.) Acrylamide (Bio-Rad, Herefordshire, UK)
- 7.5 ml 1.5 M Tris-Glycine Buffer pH 8.8 (Bio-Rad, Herefordshire, UK)
- 300 µl 10% wt/vol. SDS
- 14.1 ml distilled $H_2O$

- 300 µl 10% (wt./vol.) Ammonium Persulphate (Sigma-Aldrich, Dorset, UK)
- 50 µl TEMED (Bio-Rad, Herefordshire, UK)

All Western blot analyses were performed using the Bio-Rad mini-Protean II gel system (Bio-Rad, Herefordshire, UK) or Gene-Flow Omni-Page Wide system (for comparison of more than 10 samples: Geneflow, Stafford, UK). Transfer was achieved by the use of semi-dry transfer unit at approximately 50 mA per each 48 $cm^2$ membrane, for 2 hours at 25°C. The transfer buffer was identical to the one used for 1α-OHase. (For details about type of membrane, blocking reagent and antibodies used, for each individual protein, see the summary in Table 2.2).

#### *2.3.4.2.1 FGF-23 Stimulation of ERK-Signalling in HAoSMCs*

HAoSMCs were treated with vehicle (0.1% BSA) or FGF-23 (100ng/ml) for 10, 30 and 60 minutes. For ERK-signalling control, samples were incubated with PD98059 (Calbiochem, Damstadt, Germany) (60 µM) 30 minutes before exposure to FGF-23, according to previously published data (Perwad *et al.* 2007). Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Dorset, UK) at 50 µM for 5 minutes was used as a positive control of ERK phosphorylation. PMA Cell lysates were prepared in the presence of phosphatase inhibitors, and phosphorylated proteins were detected by ECL using rabbit anti-phospho-ERK1,2 antibodies (Cell Signal, New England Biolabs, Hitchin, UK). Equal protein loading was monitored by detection of total-ERK1,2 protein, using specific anti-rabbit antibody (Cell Signal, New England Biolabs, Hitchin, UK), for details on dilutions see the summary Table 2.2.

### 2.3.5 Protein Loading Control

β-actin is one of six actin isoforms. It is a highly conserved cytoskeletal protein involved in cell motility and cohesion. It is constitutively expressed, at high levels and it is present in most cell types. For all these reasons, β-actin is frequently used as a protein loading control in Western blot analysis. At the protein concentrations used in my study both β-actin or α-actin are directly proportional to the optical density (O.D.) of the band produced on the autoradiograph, as assessed by Image J software *(Figure 2.12).*

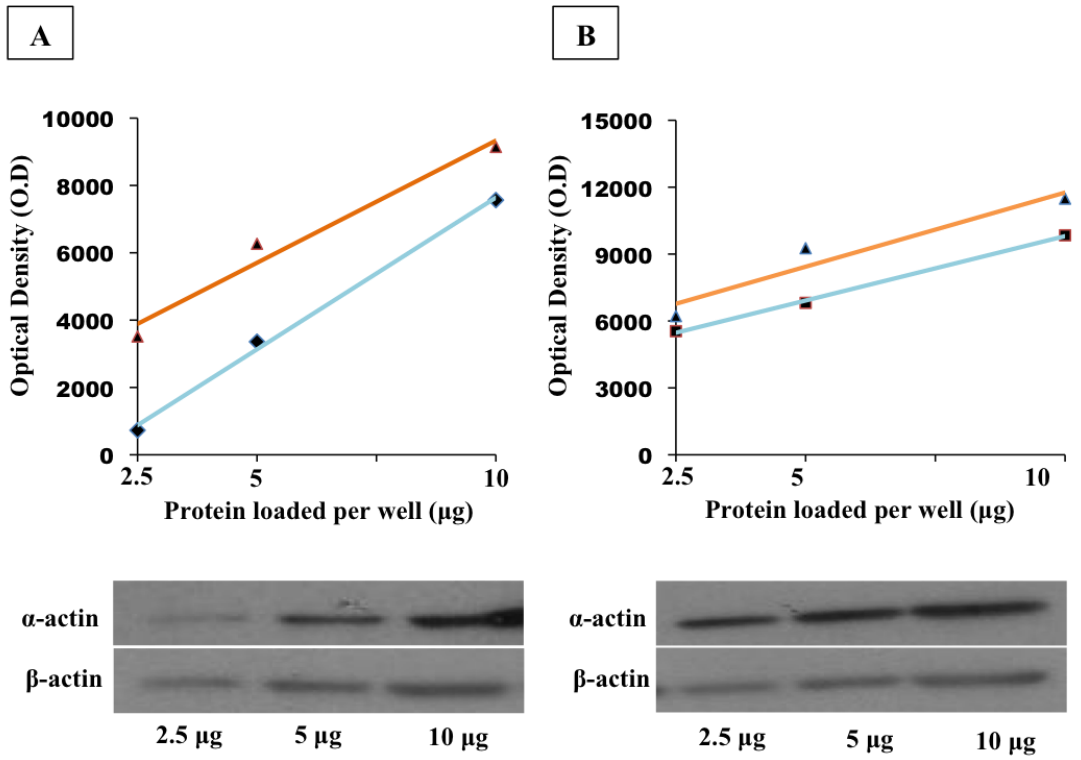


***Figure 2.12: Sample Loading for Protein Isolated from Human Aortic Smooth Muscle Cells (HAoSMCs) and Artery.*** *Increasing concentrations of protein were loaded onto 10% gels and transferred onto nitrocellulose membranes, which were probed for α-actin or β-actin. Individual band optical density (O.D.) was assessed by Image J and plotted on a graph as a function of protein concentration (2.5 µg, 5 µg, 10 µg) (A) HAoSMCs (B) Healthy human artery.*

### 2.3.6 Stripping and Re-probing of Membranes

Each filter membrane was stripped and re-probed with anti-β-actin antibody for loading control. Membranes were placed in stripping buffer (for ingredients see below) and placed in water bath at 60°C for 30 minutes, with occasional agitation. Subsequently, membranes were washed twice for 20 minutes in PBS-T and blocked in 5% (wt/vol.) non-fat milk powder Marvel for 1h. Then the standard protocol for β-actin immunoprobing was followed *(see Table 2.2).*

*Constituents of stripping buffer used for the secondary probing of proteins in Western blotting:*

- 0.7% v/v β-mercaptoethanol
- 2 % m/v SDS
- 0.98 % g/v Tris Hydrochloride

## 2.4 Immunohistochemistry

### 2.4.1 General Method

Tissues were fixed in 10% formaldehyde in water to preserve the general structure of the cytoplasm and nucleus by reacting with amino groups of proteins. The tissues were then dehydrated by passing through graded series of alcohol solutions (70%, 95%, 100%) and then placed in a non-aqueous liquid, xylene. Next, the tissues were embedded in melted paraffin, left to cool and harden, and 5 µm thick sections were cut and placed on charged slides (Superfrost Plus, BDH, Poole, UK) the fixation, embedding and cutting of section was performed by Mr Sean James (UHCW, UK).

For immunohistochemistry, sections were de-parafinised by placing slides in two changes of xylene and hydrated by sequentially immersing in 100% ethanol, 90%

ethanol and distilled water. Antigen retrieval of sections was achieved using citrate pH 6.0 (Dako, Ely, UK) and pressure-cooker, for 2min at 121°C (103 kPa).

### 2.4.2 Immunohistochemical Detection of 1α-OHase Protein in Human Artery

Immunohistochemistry for 1α-OHase was optimised using commercially available Sheep Specific HRP-DAB Tissue Staining Kit (R&D Systems, Abingdon, UK). Sections were blocked for endogenous peroxidase activity with hydrogen peroxide (3%) in methanol for 10 minutes and the washed with TBS-T, pH 7.6. Sections were incubated in 10% donkey serum for 20 minutes. The sections were then incubated with avidin blocking reagent for 15 minutes, rinsed with TBS-T and incubated with the biotin-blocking reagent for further 15 minutes. 1α-OHase antibody 1:150 (The Binding Site, Birmingham, UK) in TBS-T (0.1%) was applied and left overnight at 4°C. Sections were washed with TBS-T (0.1%) for 5 minutes between each step. Sections were incubated with ABC solution and washed with TBS-T (0.1%). The localisation of protein was visualised using 3,3-diaminobenzidine (DAB), a chromogenic substrate for peroxidase, which allowed observing permanent brown-coloured deposit at the sight of reaction. The nuclei were counterstained with Mayer's haematoxylin for 5 seconds, washed with distilled water. Section were processed through two ethanol washes and xylene and were mounted in the xylene based dibutyl polystyrene (DPX, Raymond Lamb, London, UK). Slides were scanned and images were captured using Panoramic Viewer software.

Control sections included omission of primary antibody. Data showing effective neutralisation of 1α-OHase mouse antiserum which has been used in our experiments has been published previously (Zehnder *et al.* 1999).

### 2.4.3 Immunohistochemical Detection of 24-OHase, VDR, RUNX-2, ALP and Sclerostin Protein in Human Artery

Immunohistochemistry for 24-OHase, VDR, RUNX-2, ALP and sclerostin was performed using a Mouse/Rabbit Specific HRP-DAB Novolink Polymer Detection Tissue Staining Kit (Leica, Milton Keynes, UK) according to manufacturers instructions, for details on specific antibody dilution see *Table 2.2*. Slides were scanned and images were captured using Panoramic Viewer software. RUNX-2 antibody clone AS110 (Millipore, Dundee, UK) which has previously been optimised to detect RUNX-2 protein in tissue sections was used (note: different to RUNX-2 antibody used in Western blot analysis, see *Table 2.2).*

Control sections included omission of primary antibody. Neutralisation of VDR anti-serum has been demonstrated in the past (Khadzkou *et al.* 2006).

## 2.5 Detailed Summary of Western Blot and Immunohistochemistry Protocols

***Table 2.2 Detailed summary of Western blot and immunohistochemistry protocols.*** *1α-Hydroxylase (1α-OHase), 24-Hydroxlase (24-OHase), Vitamin D Receptor (VDR), Alkaline Phosphatase (ALP), FGF Receptor (FGFR), Runt-related Transcription Factor 2 (RUNX-2); primary antibody (1°Ab), secondary antibody (2°Ab), not-applicable (N/A), Tris-buffered saline with 0.1% tween (TBS-T), Horse Radish Peroxidase – 3,3-Diaminobenzidin (HRP-DAB).*

| ANTIBODY | COMPANY | WESTERN BLOT – dilution and conditions | IMMUNOHISTO-CHEMISTRY dilution and conditions |
|---|---|---|---|
| 1α-OHase IgG fraction | The Binding Site, Birmingham, UK | 1° Ab. Sheep polyclonal 1:500 in PBST 0.05% at 4°C overnight<br>2° Ab. Donkey anti-sheep peroxidase conjugated 1:20,000 in PBST 0.05%, at 25°C for 1 hour<br>PVDF membrane | 1°Ab. 1:150 in PBS<br>Cell and Tissue Staining Kit, Sheep, HRP-DAB, (R&D Systems, UK) |
| 24-OHase | Sigma- | 1°Ab. Mouse monoclonal 1:500 | 1°Ab. 1:200 in PBS |

| | | | |
|---|---|---|---|
| clone 1F8 | Aldrich, Dorset, UK | in PBST 0.05% at 4°C overnight<br>2°Ab. Anti-mouse, 1:5,000 in PBST 0.05%, 5% milk at 25°C for 1 hour<br>PVDF membrane | Novolink Polymer Detection HRP-DAB System, (Leica, Milton Keynes, UK) |
| VDR<br>clone C-20 | Santa Cruz, Wembley, UK | 1°Ab. Rabbit polyclonal 1:500 in Animal Serum Free Blocker (ASFB) at 4°C overnight<br>2°Ab. Anti-rabbit, 1:5,000 in ASFB at 25°C for 1 hour<br>Nitrocellulose membrane | 1°Ab. 1:200 in PBS<br>Novolink Polymer Detection HRP-DAB System, Leica |
| RUNX-2 | Santa Cruz Biotechnology Inc., Santa Cruz, USA | 1°Ab. Mouse monoclonal 1:1,000 in 5% milk at 4°C overnight<br>2°Ab. Anti-mouse 1:5,000 in 5% milk at 25°C for 1hour<br>Nitrocellulose membrane | N/A |
| RUNX-2<br>clone AS110 | Millipore, Dundee, UK | N/A | 1°Ab. 1:200 in TBS-T<br>Novolink Polymer Detection HRP-DAB System, (Leica, Milton Keynes, UK) |
| Klotho | ABcam, Cambridge, UK | 1°Ab. Rabbit polyclonal 1:1,000 in 1% milk at 4°C overnight.<br>2°Ab. Anti-mouse 1:5000 in 5% milk at 25°C for 1hour<br>Nitrocellulose membrane | N/A |
| α-Actin<br>clone 1A4 | Sigma Aldrich, Dorset, UK | 1°Ab. Mouse monoclonal 1:2,000 in 5% BSA 0.1% PBST at 4°C overnight<br>Nitrocellulose membrane | N/A |
| β-actin – loading control | Cell Signal, New England Biolabs, Hitchin, UK | 1°Ab. Rabbit polyclonal 1:1,000 in 5% BSA 0.1% PBST at 4°C overnight<br>2°Ab. Anti-rabbit 1:5000 in 5% milk at 25°C for 1 hour<br>Any membrane | N/A |
| ERK-1/2 | Cell Signal, New England Biolabs, Hitchin, UK | 1°Ab. Rabbit polyclonal 1:2,000 in 5% BSA 0.1% PBST at 4°C overnight<br>2°Ab. Anti-rabbit 1:5,000 in 5% milk at 25°C for 1 hour<br>Nitrocellulose membrane | N/A |
| p-ERK-1/2 | Cell Signal, New | 1°Ab. Rabbit polyclonal 1:1,000 in 5% BSA 0.1% PBST at 4°C | N/A |

| | | | |
|---|---|---|---|
| | England Biolabs, Hitchin, UK | overnight<br>2°Ab. Anti-rabbit 1:5000 in 5% milk at 25°C for 1 hour<br>Nitrocellulose membrane | |
| Sclerostin | Santa-Cruz Biotechnology Inc., Santa Cruz, USA | N/A | 1°Ab. 1:40 in TBS-T<br>Novolink Polymer Detection HRP-DAB System, (Leica, Milton Keynes, UK) |
| ALP | ABcam, Cambridge, UK | 1°Ab. Rabbit polyclonal 1:500 in 1% milk at 4°C overnight.<br>2°Ab. Anti-mouse 1:5,000 in 5% milk at 25°C for 1hour<br>PVDF membrane | 1°Ab. 1:50 in TBS-T<br>Novolink Polymer Detection HRP-DAB System, (Leica, Milton Keynes, UK) |
| FGFR-1 (Fgl) clone C15 | Santa Cruz Biotechnology Inc., Santa Cruz, USA | 1°Ab. Rabbit polyclonal 1:1,000 in 5% BSA 0.1% PBST at 4°C overnight<br>2°Ab. Anti-rabbit 1:5000 in 5% milk at 25°C for 1 hour<br>Nitrocellulose membrane | N/A |
| FGFR-3 clone C15 | Santa Cruz Biotechnology Inc., Santa Cruz, USA | 1°Ab. Rabbit polyclonal 1:1,000 in 5% BSA 0.1% PBST at 4°C overnight<br>2°Ab. Anti-rabbit 1:5000 in 5% milk at 25°C for 1 hour<br>Nitrocellulose membrane | N/A |

## 2.6 Assessment of Calcium Deposition in Arterial Sections using Alizarin Red Staining

Immunohistochemistry sections were de-parafinised as described before (2.4.1). 2% Alizarin Red S (Sigma-Aldrich, Dorset, UK) pH 4.2. The tissue sections were stained for 2 minutes and washed with distilled water. Sections were dehydrated in 100% acetone (20 dips) and in acetone-xylene (1:1) solution (20 dips), followed by 5 minutes in xylene solution prior to mounting. Areas of calcium deposition stained red. Slides were scanned and images were captured using Panoramic Viewer software.

## 2.7 RNA Isolation and Analysis

### 2.7.1 Principle

The principle of extracting total mRNA based on the one step-acid guanidinium method was first described by Chomczynski and Sacchi (Chomczynski and Sacchi 1987). In this study, total RNA was extracted from cells using the QiagenRNeasy kit (Qiagen, Crawley, UK), and procedure was carried out according to a modified protocol provided by the manufacturer.

### 2.7.2 RNA Extraction Procedure

Cells were washed twice in PBS and 2 ml of RNA lysis buffer was added per 73 $cm^3$ flask, with all steps performed on ice as follows. Cells were then gently scraped and lysate was transferred to microfuge tubes. Arterial rings (0.2-0.3 $cm^3$) were placed in large Eppendorfs, kept on ice and 150-200 µl of RNA lysis buffer was added. Small plastic pestles were used to manually grind the tissue.

1 volume of 70% vol./vol. molecular grade ethanol (Sigma-Aldrich, Dorset, UK) was added and sample was transferred to RNeasy column, and then centrifuged for 15 seconds at 9000 g. RNA remained bound to the filter (silica-based membrane with selective binding properties allows isolation of up to 100 µg of RNA longer than 200 bases) and the flow-through was discarded. Subsequently, two different buffers: RW1 (containing guanidine thiocyanate) and RPE (high salt buffer) were used to wash and purify the RNA fraction bound to the filter. RNA was eluted by the addition of 30 µl of RNase free water to the filter and centrifuging the sample for 1 minute at 9000 g. Samples were stored at -20 °C.

The concentration of the isolated RNA was assessed by measuring spectrophotometric absorbance at 260 nm (ND-1000 Spectrophotometer) and calculated against 1 optical density (OD) unit at 260 nm being equivalent to 40 µg of RNA in 1 ml. The ratio of absorbance at 260 nm to 280 nm (260/280) above 1.8 was considered as an RNA sample pure of DNA contamination. The usual RNA concentration from HAoSMCs was 200 ng/µl and for tissues, 400 ng/µl.

### 2.7.3 RNA Reverse Transcription (RT-PCR)

Reactions were set up in a final volume of 20 µl. 500 ng of total RNA, 50 ng/ml random primers, 0.1 mM dNTP mix was combined and the reaction brought to 13 µl with nuclease-free $H_2O$. Samples were heated at 65°C for 5 minutes, and then placed on ice. Subsequently, a RT master-mix was made-up containing 1x buffer, 0.1M DTT, 40 u/µl RNAse inhibitor and 200 u/µl of Superscript Reverse Transcriptase III. 7 µl of the master mix was added to each sample to bring the total volume to 20µl. Samples were mixed and incubated at 25°C for 5 minutes, 50°C for 1 hour, 70°C for 15 minutes and cooled to 4°C. cDNA was stored at -20°C.

### 2.7.4 Polymerase Chain Reaction

#### *2.7.4.1 Principle*

The polymerase chain reaction is a process in which a product from one cycle of amplification serves as a substrate in the following cycle. The amount of product increases exponentially, and provided ideal theoretical conditions unrestricted by the

substrate limitation, the amount of product doubles during each cycle according to the equation:

$$N = N_0 2^n$$

***Equation 1: Product formation in PCR reaction***. *Where N is a number of amplified molecules; $N_0$ is an initial number of molecules and n is a number of amplification cycles.*

PCR was carried out using the cDNA obtained by reverse transcription at an appropriate dilution with the appropriate primers (Zehnder *et al.* 2008) (*Table 2.3*).

***Table 2.3: Primers for RNA detection and quantification.*** *Forward and reverse PCR primer sequences for 24-hydroxylase (24-OHase), 1α-hydroxylase (1α-OHase) and vitamin D receptor (VDR); F-forward, R-reverse.*

| PRIMER | Sequence (5'→3') | Product Size (bp) |
|---|---|---|
| 24-OHase (F) | CCCACTAGCACCTCGTACCAAC | 485 |
| 24-OHase (R) | CGTAGCCTTCTTTGCGGTAGTC | |
| 1α-OHase (F) | ACGCTGTTGACCATGGC | 542 |
| 1α-OHase (R) | GTGACACAGAGTGACCAGCATAT | |
| VDR (F) | CGCTCCAATGAGTCCTTCACC | 421 |
| VDR (R) | GCTTCATGCTGCACTCAGGC | |

A 40µl PCR reaction was set up with 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2mM dNTPs, 0.2 pM forward and reverse primers, 1 u Taq DNA polymerase and 2 µl cDNA. The reactions were made up to 40 µl with nuclease free water and PCR was performed in a thermal cycler. HKC-8 or kidney cDNA were used as controls (VDR, 1α-OHase and 24-OHase) (Bland *et al.* 1999), water was used as a negative control.

***Table 2.4: RT-PCR cycling conditions.***

| TEMPERATURE | TIME | STEP | CYCLES |
|---|---|---|---|
| 95°C | 5 minutes | Initial denaturation | |
| 95°C | 1 minute | Denaturation | |
| 59°C | 1 minute | Annealing | 35 cycles |
| 72°C | 1 minute | Extension | |
| 72°C | 5 minutes | Extension | |
| 4°C | Hold | Cooling | |

### 2.7.5 DNA Gel Electrophoresis

DNA separation was achieved using agarose gel electrophoresis. 1.5% (wt/vol.) gel was made up with TBE buffer containing ethidium bromide at 0.2μg/ml. 5xDNA loading buffer (Bioline, London, UK) was added to a final concentration of 1x to each sample. DNA Hyperladder I (Bioline, London, UK) was used as a size marker (5 μl). PCR bands were visualised under UV light and images captured using gel doc system.

### 2.7.6 Real Time RT-PCR

#### *2.7.6.1 Principle*

The main difference between standard RT-PCR and Real-Time RT-PCR is that the latter allows quantification of the product in each individual cycle as the reaction progresses. It takes advantage of the 5' nuclease activity of AmpliTaq Gold DNA

polymerase, which removes any double stranded DNA and cleaves the TaqMan gene specific probe, which is hybridised with the target cDNA that is amplified. The probe contains a reporter dye at its 5' end and a quencher at its 3' end. The quencher suppresses fluorescence emitted by the dye. As the complementary strand is synthesised, cleavage of the probe separates the reporter dye from the quencher, resulting in increased fluorescence of the reporter (Livak *et al.* 1995). The probe fragments are blocked at 3' ends and removed from the target and the polymerisation of the strand continues.

As amplification proceeds, the fluorescence accumulation is captured by the instrument after every cycle, and is translated into a Real-Time RT-PCR graph. There are three amplification stages: exponential, linear and plateau. In the exponential phase the reagents are in the abundance, which allows the most precise and accurate data for quantitation *(Figure 2.14A).*

The Comparative Ct Method was used to analyse the results. In the exponential phase two values are calculated: the Threshold line (which is the level of detection at which the reaction reaches the level of fluorescent intensity above background) and the Ct (threshold cycle for amplification, which is the PCR cycle at which the sample reaches the Threshold line; it is a relative measure of concentration of target in the Real-Time PCR reaction) *(Figure 2.14B).*

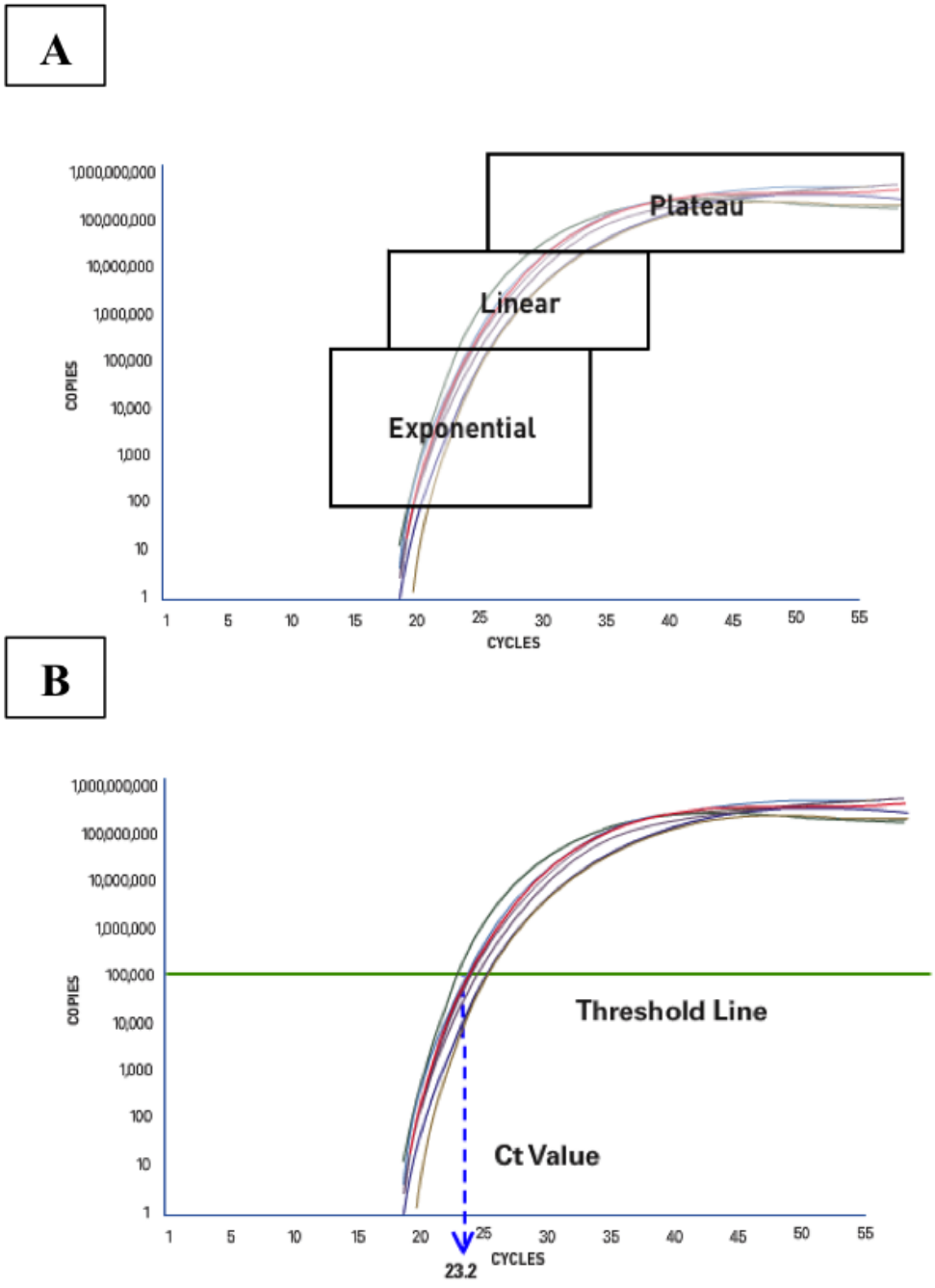


***Figure 2.13: The Real-Time RT-PCR curve***. *A) There are three amplification stages: 1-Exponential, 2-Linear, 3-Plateau. B) The PCR cycle at which the sample reaches the threshold level is called the cycle threshold, Ct and is used in downstream quantitation. Adapted from http://www.appliedbiosystems.com.*

Arithmetic Formulae:

**ΔCt = Ct of the target gene minus Ct of the housekeeping gene**

**ΔΔCt = ΔCt of the target amplification minus ΔCt of the reference amplification.**

The amount of target, normalised to an endogenous reference is given by the Equation 2:

$$2^{-\Delta\Delta Ct}$$

***Equation 2: The amount of target normalized to endogenous reference****. Where ΔCt = Ct of the target gene - Ct of the housekeeping gene; ΔΔCt = ΔCt of the target amplification - ΔCt of the reference amplification.*

This means that the amount of target mRNA is two times (each cycle one copy from one template) the power relative to cycle number compared to the housekeeping gene and to the reference. This formula expresses the fold increase or decrease of target (mRNA) compared to the reference. In this study, all experiments were multiplexed (multiple cDNA targets were simultaneously amplified using a single sample) and the mRNA of interest were normalised to 18S rRNA.

### *2.7.6.2 Method for Real-Time RT-PCR Analysis*

Samples were prepared in 96-well micro-plates, in the total volume of 25 µl, that included 23 µl of mastermix (12.5 µl TaqMan buffer, 1.25 µl gene specific TaqMan probe, 1.25 µl of 18S probe, 8 µl $H_2O$) and 2 µl of sample cDNA. The plate was sealed using MicroAmo optical adhesive film (Applied Biosystems, Bedford, UK)

and centrifuged at 600 g for 30 seconds. 7500 Systems software (Applied Biosystems, Bedford, UK) was used for detection of the fluorescence signal.

### *2.7.6.3 The TaqMan Probe Information:*

The primers have been designed previously by (Zehnder *et al.* 2008).

*CYP27B1*(1α-Hydroxylase, Hs00168017_m1)

- Primer sequence:

  FAM reporter **5' [bp]-TTGCAATTCAAGCTCTGCCAGGCG-[bp] 3'** quencher

*CYP24A1* (24-Hydroxylase, Hs00989013_g1)

- Primer sequence:

  FAM reporter **5'[bp]-ACTACCGCAAAGAAGGCTACGGGCTG-[bp] 3'** quencher

*VDR* (Vitamin D Receptor, Hs01045847_m1)

- Primer sequence:

  FAM reporter **5'[bp]-AAGGCACTATTCACCTGCCCCTTCAA-[bp] 3'** quencher

18S

- Reporter dye: VIC

## 2.8 Statistical Analysis

The data were presented graphically and analysed statistically using Microsoft Excel 2010, Prism 7.0 and SPSS 19 software.

### 2.8.1 The Two Sample (Unpaired) Student's t-Test

In this study all data were tested for normality and the non-normally distributed data were normalised by logarithmic transformation (Ln). The unpaired parametric Student's t-test was used to analyse the samples from normally distributed populations with the same variances, in order to assess whether the means of two different groups were greater than what can be attributed to random sampling variation. In graphical representation, dot plots were used, where each dot depicted individual patient sample (the optimal and informative approach to show the variation among the values) and its distance from the mean (where mean was depicted by a bar).

### 2.8.2 One-way Analysis of Variance (ANOVA) and Tukey-Kramer Testing

Statistical analysis of more than two experimental groups from normally distributed populations was performed using one-way ANOVA (parametric), with post-hoc testing using Tukey-Kramer multiple comparison post-test. Tukey-Kramer testing was important to confirm the results of ANOVA, as no single study can support a whole series of hypotheses.

### 2.8.3 Probability

p values (meaning the probability of observing the data when the null hypothesis is true) were calculated, where $p < 0.05$ was considered as statistically significant.

### 2.8.4 Standard Deviation and Standard Error of the Mean

Standard Deviation (SD) describes the average spread of observations around the mean. The Standard Error of the Mean (SEM) is the standard deviation of the error in the sample mean relative to the true mean. SEM can be calculated by the sample estimate of the population SD divided by the square root of the sample size, assuming statistical independence of the values in the sample.

### 2.8.5 Real-Time RT-PCR Statistical Analysis

Real-Time RT-PCR data were depicted as fold changes, which were calculated using ΔΔCt method (2.7.6). The experimental values were expressed as relative to the control value of 1. It is important to note, that the error bars (SEM) were omitted from all Real-Time RT-PCR graphs, as the representation of data with ΔΔCt method implies an exponential transformation ($2^{-\Delta\Delta Ct}$) and since the error bars originated at the ΔΔCt step, they would be asymmetrical. Consequently, ΔCt values were used for statistical analysis (one-way ANOVA with Tukey-Kramer post-tests).

### 2.8.6 Product moment correlation coefficient: r

The product moment correlation coefficient (Pearson's r) test assesses the strength of the straight line between the continuous variables. Pearson's r between variables

is defined as the covariance of the variables divided by the product of their SDs Strength of correlation can be interpreted as negative: -1.0 to -0.1 and positive 0.1-1.0; 0 indicates no correlation (Belle 2004).

### 2.8.7 Coefficient of determination: $R^2$

$R^2$ is the square of the correlation coefficient and describes the fraction of variance between the two dependent variables. It informs how good is one variable in predicting another, if $R^2=1$, then if one value is given, another value can be perfectly predicted. If $R^2=0$, then even if both values are given, the prediction of any value is impossible.

## 2.9 Web-based Resources

The gene DNA sequence searches for primer design were performed using Blast engine, www.ncbi.nlm.nih.gov. The U.S. National Library of Medicine (PubMed), at www.ncbi.nlm.nih.gov/pubmed and MEDLINE, at http://ovidsp.tx.ovid.com were the main sources of literature.

# Chapter 3

# Expression of the Vitamin D System in Human Artery

## 3.1 Background

The discovery of the VDR in 1969 was followed by the emergence of multiple reports demonstrating the presence of the receptor in over 30 tissues/organs of human body (Freake *et al.* 1981, Holick *et al.* 1987, Karmali *et al.* 1989, Kuroki 1985, Norman A. W. 2006, Ohta *et al.* 1985, Reitsma *et al.* 1983, Simpson *et al.* 1987). VDR protein and mRNA was also shown to be present in human vasculature, in endothelial cells, smooth muscle cells and in cardiomyocytes (Zehnder, Lubczanska 2012 – unpublished, part of other research project, Wu-Wong *et al.* 2006a).

Recent studies suggest that conversion of 25(OH)D to 1,25$(OH)_2$D in a number of tissues, independent of renal conversion may play a role in survival. Observational studies demonstrated that survival rate in patients on dialysis is higher in patients on VDR activation therapy (Kalantar-Zadeh *et al.* 2006, Teng *et al.* 2005, Tentori *et al.* 2006, Wolf and Thadhani 2007). Further, comparison of survival in patients where two different VDRA therapeutic regimes were used: paricalcitol and calcitriol demonstrated that the mortality rate was 16% lower using paricalcitol versus

calcitriol (Teng *et al.* 2003). VDR activators have also been associated with beneficial cardiovascular outcomes in patients with CKD (Shroff R. C. *et al.* 2008).

Previous reports suggest that VDR binding in heart may have beneficial antihypertrophic effects (Zittermann *et al.* 2005). This was confirmed in a global *VDR*-knockout mouse model, where mice with deleted *VDR* exhibited cardiac hypertrophy (Rahman *et al.* 2007). A recent study by Chen and colleagues investigated the effect of targeted deletion of *VDR* gene in rat cardiomyocytes and compared the effects to the ones previously described in full *VDR*-knockout mice (Chen S. *et al.* 2011, Rahman *et al.* 2007, Xiang *et al.* 2005). All studies reported an increased mRNA and protein levels of modulatory calcineurin inhibitory protein 1 (MCIP1) in all *VDR*-knock out animals. Expression of MCIP1 was shown to correlate with calcineurin/nuclear factor of activated T cells (NFAT) signalling in the heart and its knockout in mice resulted in reduction in cardiac hypertrophy (Sanna *et al.* 2006, Vega *et al.* 2003), suggesting that there are vasculature-specific vitamin D dependent regulatory pathways.

Recent studies in vasculature demonstrated that VSMC vitamin D hormonal system can be regulated by PTH and estrogenic compounds (Somjen *et al.* 2005). VDR-signalling in vascular endothelial cells was shown to be regulated also by inflammatory cytokines (Zehnder *et al.* 2002b). Irrespectively of all evidence, some still argue that vitamin D hormonal system is not present in extra-renal tissues (Vanhooke *et al.* 2006).

Evidence suggests expression and activation of the VDR is protective/maybe beneficial and prevent CVD and CV calcification in renal patients. Despite this people still dispute whether the vitamin D signalling system exists in vascular tissue. I therefore wanted to investigate the expression of components of the vitamin D

system in healthy and diseased human arteries and more specifically in VSMC. This would also allow me to determine the suitability of VSMC as a model system in which to further investigate the regulation of vitamin D signalling in the artery. Human healthy and CKD epigastric and renal arteries were obtained as described in chapter 2.1. mRNA expression was assessed by real-time RT-PCR, proteins identification and level of expression were determined by Western blot analyses, and localized by immunohistochemistry in tissues sections.

## 3.2 Results

### 3.2.1 Expression of the Vitamin D system in Healthy Human Artery and Vascular Smooth Muscle Cells

#### *3.2.1.1 Expression of the Vitamin D Receptor (VDR)*

RT-PCR analyses revealed that VDR mRNA was detected in HAoSMCs and that it was a similar size transcript to that seen in HKC-8 (human kidney proximal tubule cells were used as control) *(Figure 3.1A).* Western analyses were carried out using proteins isolated from HAoSMCs and normal human artery (HKC-8 and normal human kidney were used as controls). In both, HAoSMCs and HKC-8 cells a doublet was detected (approximately 48 kDa and 50 kDa in HAoSMCs and 50 kDa and 52 kDa in HKC-8) (*Figure 3.1B,* as discussed in more detail in *2.3.4.1.1*). Western blot analysis of protein isolated from normal human artery demonstrated presence of one strong band of about 50 kDa (*Figure 3.1C*). The higher molecular band (52 kDa) appeared in all tested cells. Although expression in HAoSMCs was lower and longer

exposure times were required. Similar size bands were observed in normal human kidney.

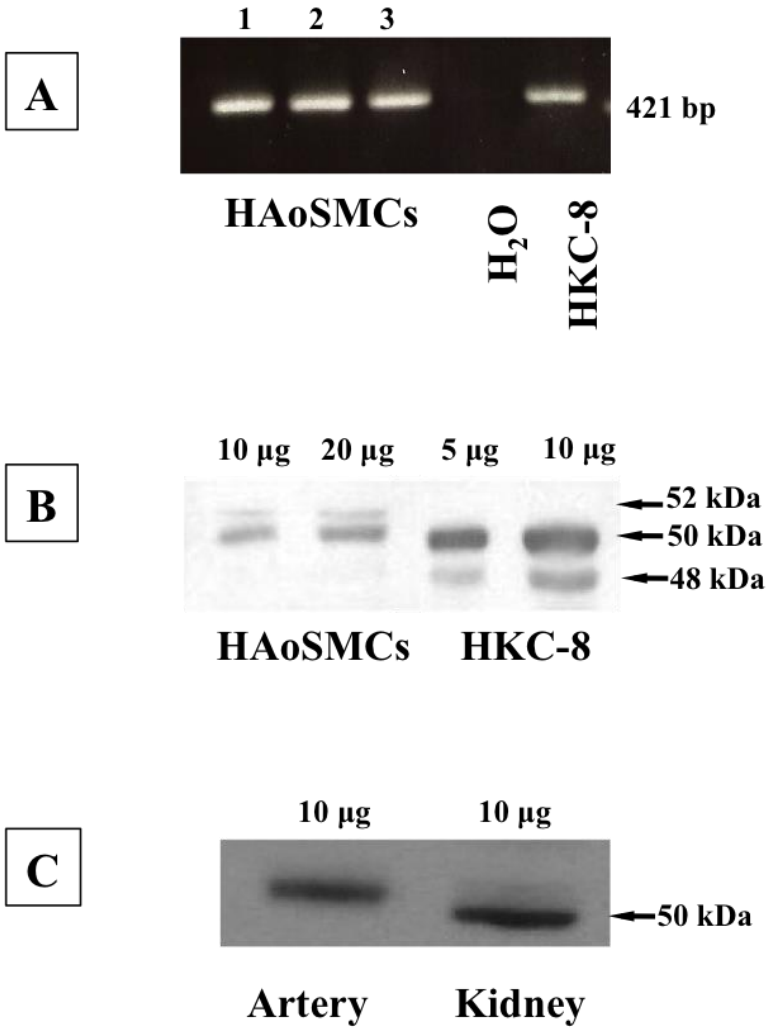


***Figure 3.1: The expression of Vitamin D Receptor (VDR) mRNA and protein in Human Aortic Smooth Muscle Cells (HAoSMCs) and normal human artery****. (A) RT-PCR analyses using primers specific for VDR mRNA, in both HAoSMCs from three (1,2,3) different donors; water ($H_20$) negative control. Human kidney proximal tubule cells (HKC-8) as a positive control. The expected PCR product of 421 bp was observed. (B) Western blot analysis of HAoSMC protein (10 µg/lane and 20 µg/lane) and HKC-8 protein (5 µg/lane and 10 µg/lane) (C) and normal human artery protein (10 µg/lane); kidney as control, using rabbit polyclonal antibody specific for VDR (C-20)).*

### *3.2.1.2 Expression of the 25-Hydroxyvitamin D 1α-Hydroxylase (1α-OHase, CYP27B1)*

HKC-8 cells and cortical region of healthy human kidney tissue have been shown previously to express 1α-OHase (Bland *et al.* 1999, Zehnder *et al.* 1999) and I have used them as positive controls. RT-PCR analyses confirmed the presence of *CYP27B1* mRNA in HAoSMCs revealing similar size transcripts to those seen in HKC-8 (*Figure 3.2A*). Western blot analysis of protein isolated from HAoSMCs demonstrated the presence of two bands of approximately 54 and 56 kDa (where the 56 kDa band always appeared fainter than the 54 kDa band). Protein isolated form HKC-8 were used as positive control previously shown to express 1α-OHase (Bland *et al.* 1999) (only 54 kDa band was detected) (*Figure 3.2B*). In artery, the appearance of the doublet was even more prominent and bands were similar in size to the ones detected in kidney *(Figure 3.2C).* As shown in the methods section, the specificity of antiserum was assessed by its pre-incubation with a 100-fold excess of immunising peptide. This resulted in neutralisation of all bands in HAoSMCs, artery, and significant reduction in the kidney *(Figure 2.11).*

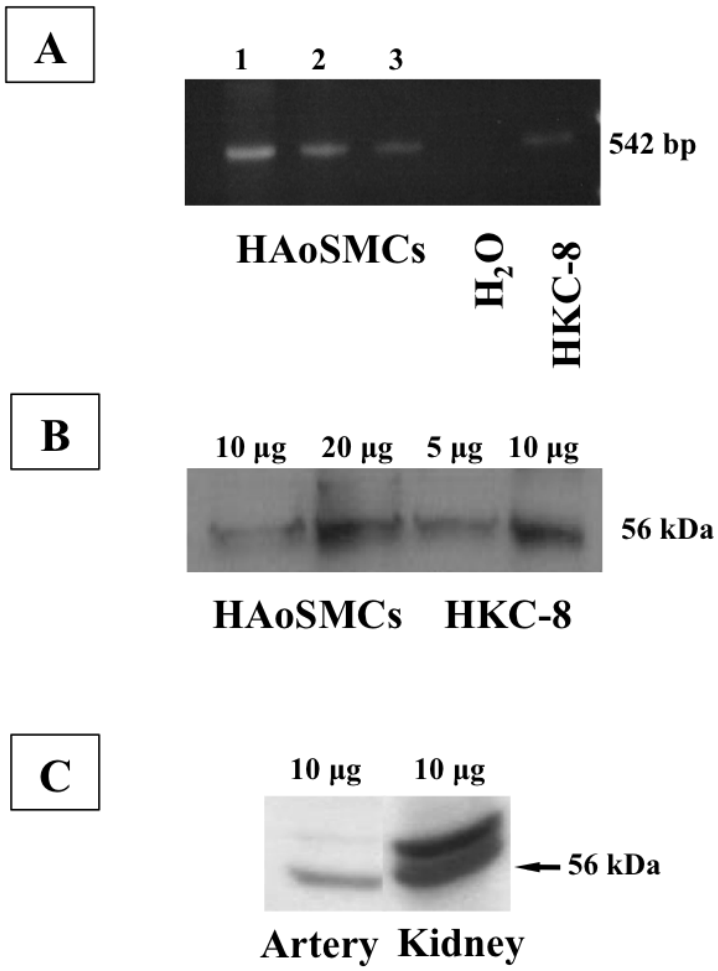


***Figure 3.2: The expression of 1α-Hydroxylase mRNA (CYP27B1) and protein (1α-OHase) in Human Aortic Smooth Muscle Cells (HAoSMCs) and normal human artery****. (A) RT-PCR analyses using primers specific for CYP27B1 mRNA, in both HAoSMCs from three (1,2,3) different donors; water ($H_20$) negative control; human kidney proximal tubule cells (HKC-8) as control. The expected PCR product of 542 bp was observed (B) Western blot analysis of HAoSMC protein (10 µg/lane and 20 µg/lane) and HKC-8 protein (5 µg/lane and 10 µg/lane) (C) and normal human artery protein (10 µg/lane); kidney as positive control using antibody specific for 1α-OHase, confirmed the presence of 1α-OHase protein both in HAoSMCs and normal human artery (band detected: approximately 56 kDa).*

#### *3.2.1.3 Expression of the 24-Hydroxylase (24-OHase, CYP24A1)*

The presence of 24-OHase protein and mRNA has been shown throughout the kidney and in other non-renal vitamin D responsive tissue (Akeno *et al.* 1997, Kumar *et al.* 1994, Lechner *et al.* 2007, Schuster *et al.* 2001), but not in human vasculature

My results have demonstrated for the first time that both HAoSMCs and healthy human artery express 24-OHase. RT-PCR analyses confirmed the presence of *CYP24A1* mRNA in HAoSMCs, where the PCR product was the same size as the one seen in HKC-8 *(Figure 3.3A).* Western blot analyses carried out using proteins isolated from HAoSMCs and normal human artery, demonstrated the presence of a single band of approximately 56 kDa. A similar size band was also observed in HKC-8 and human kidney (*Figure 3.3B* and 3.3C). In tissues, protein expression appeared to be at similar level. Conversely, protein expression of 24-OHase appeared to be at a lower level in HAoSMCs compared to expression in HKC-8. Human artery appeared to express 24-OHase protein at a level similar to that seen in the kidney, but HAoSMC expression was lower than that seen in HKC-8 cells *(Figure 3.3C).*

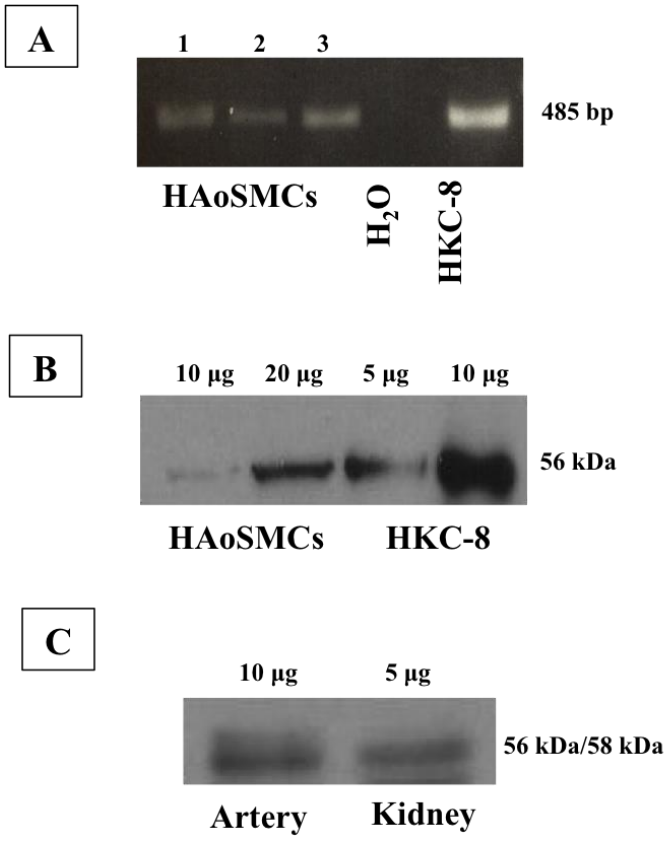


***Figure 3.3: Expression of the 24-Hydroxylase mRNA (CYP24A1) and protein (24-OHase) in Human Aortic Smooth Muscle Cells (HAoSMCs) and human artery from healthy individuals****. (A) RT-PCR analyses using primers specific for 24-OHase mRNA, in both HAoSMCs from three (1,2,3) different donors; water ($H_20$) negative*

*control; human kidney proximal tubule cells (HKC-8) as positive control. The expected PCR product of 485 bp was observed. (B) Western blot analysis of HAoSMC protein (10 μg/lane and 20 μg/lane) and HKC-8 protein (5 μg/lane and 10 μg/lane) (C) and normal human artery protein (10 μg/lane); kidney as control, using mouse monoclonal antibody specific for 24-OHase (1F8), confirmed the presence of 24-hydroxylase protein both in HAoSMCs and normal human artery (band detected: approximately 56 kDa).*

The vascular distribution and cellular abundance of VDR, 1α-OHase and 24-OHase protein in human kidney and human arteries from healthy individuals donating a kidney were assessed by immunohistochemistry of formalin fixed and paraffin embedded sections. Strong immunoreactivity was observed for both VDR and 1α-OHase, with relatively low staining for 24-OHase (positive staining brown) in the medial layer of the artery, with visible staining of individual SMCs *(Figure 3.4).* Consistent with previous findings in kidney (Kumar *et al.* 1994, Zehnder *et al.* 1999), staining for 1α-OHase and 24-OHase in VSMCs was cytoplasmic. The VDR appeared to be present both in the cytoplasm and in the nuclei. Further, VDR, 1α-OHase and 24-OHase staining was also present in the endothelium (single-cell layer on the inner periphery of the artery, 200X *Figure 3.4*). Endothelial presence of 1α-OHase and VDR protein and synthesis of 1,25$(OH)_2$D has been described previously in human vascular endothelial cells (Zehnder *et al.* 2002b).

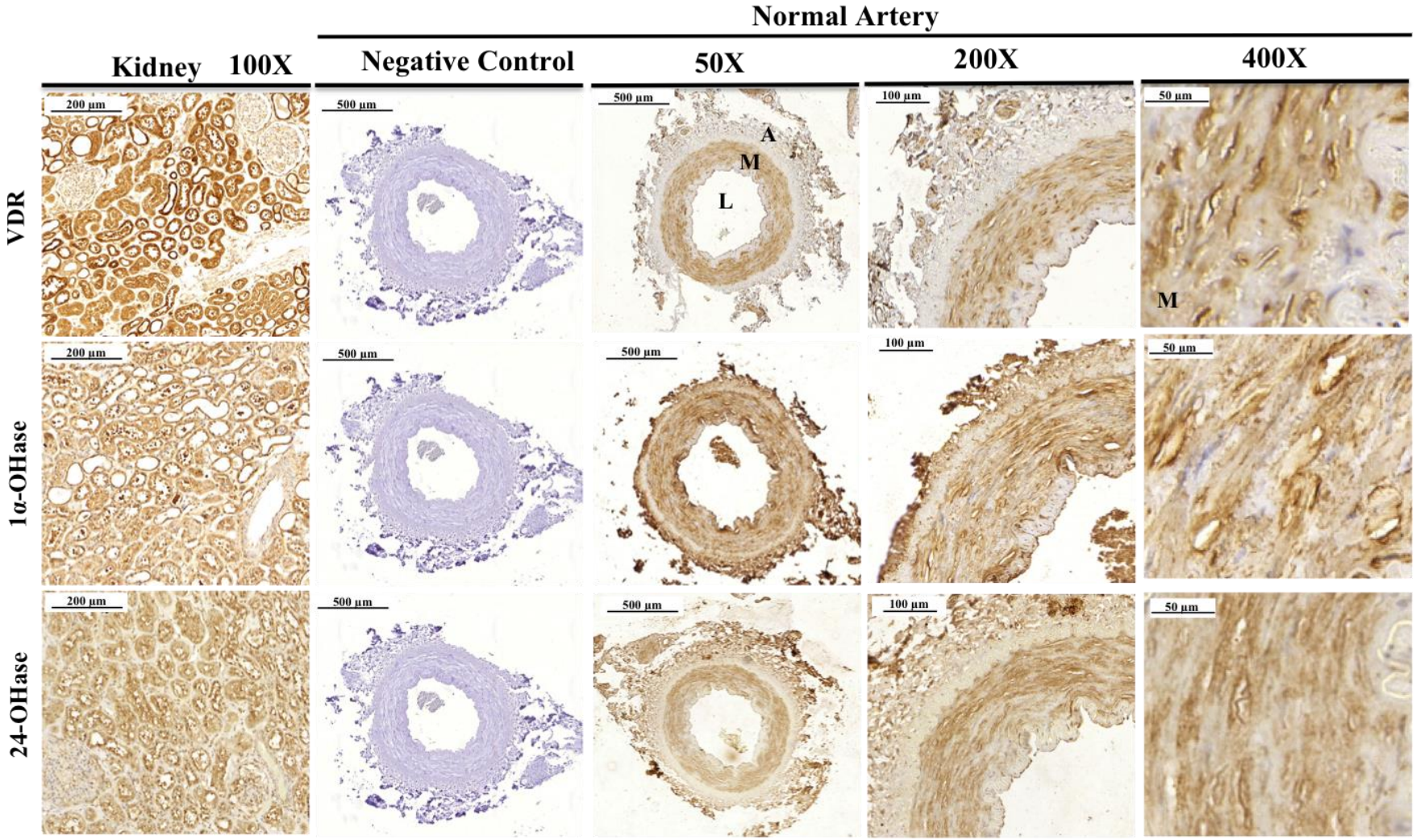


***Figure 3.4: Expression and localisation of the Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) proteins by immunohistochemistry in sections of human artery from healthy individuals.*** *Strong positive staining (brown) for all three proteins was observed in the medial (M) layer of the artery. Negative control: the primary antibody was omitted. Positive control: human kidney, cortex. Magnification: 50X, 200X and 400X. L-lumen, A-adventitia*

### 3.2.2 Comparison of Expression of the Vitamin D System in Artery from Healthy Individuals and in Patients with CKD

#### *3.2.2.1 Quantification of mRNA Expression by Real-Time RT-PCR Analyses*

RNA was extracted from 11 healthy and 17 CKD arteries (see 2.1), transcribed and real-time PCR analyses were performed using *VDR*, *CYP27B1*, *CYP24A1* or *RUNX-2*-specific probes. The patient details including age, gender, blood pressure, hypertension, diabetes, CKD stage, renal diagnosis, blood haemoglobin, serum creatinine, serum calcium, phosphate and eGFR are outlined in Table 3.1.

***Table 3.1 Summary of Demographic and Clinical Data of Patients Included in mRNA Expression Analyses (Real Time RT-PCR).*** *Chronic Kidney Disease (CKD), Haemodialysis (H), Body Mass Index (BMI), Autosomal Dominant Polycystic Kidney Disease (ADPKD).*

| Patient Characteristics | Healthy | CKD | P |
|---|---|---|---|
| Age (median, range; years) | 50 (23-70) | 54 (23-67) | *NS* |
| Male (%) | 6 (60%) | 10 (63%) | |
| BMI (mean ± SD; kg/m$^2$) | 24.2 ± 5.3 | 25.7 ± 2.8 | *NS* |
| Nicotine (n) | 4 (40%) | 9 (56%) | |
| Blood Pressure (mean ± SD; mmHg) | | | |
| Systolic | 124 ±16.8 | 132 ± 18.0 | *NS* |
| Diastolic | 74 ±15.9 | 75 ± 10.5 | *NS* |
| Hypertension (%) | 1 (10%) | 14 (88%) | |
| Diabetes (%) | 0 | 2 (13%) | |
| CKD Stage (n) | | | |
| 5 | 0 | 4 | |
| 5D | 0 | 12 | |
| Renal diagnosis (n) | | | |
| ADPKD | - | 5 | |
| Glomerulonephritis | - | 5 | |
| Diabetes | - | 2 | |
| Other | - | 4 | |
| Laboratory (mean ± SD) | | | |
| Haemoglobin blood (g/dL) | 12.0 ± 1.6 | 11.6 ± 1.6 | *NS* |
| Creatinine serum (μM) | 87 ± 32.3 | 674 ± 296.9 | *<0.0001* |
| Calcium (mM) | 2.09 ± 0.12 | 2.3 ± 0.12 | *<0.01* |
| Phosphate (mM) | 1.2 ± 0.15 | 1.6 ± 0.47 | *<0.05* |
| eGFR (ml/min/1.73m$^2$) | 84.7 ± 22.9 | 9.4 ± 5.0 | *<0.0001* |

mRNA expression was corrected by 18S rRNA expression levels and presented graphically as a percentage, relative to normal untreated control (100%). Expression of *VDR* mRNA in CKD arteries was significantly reduced by 68%, compared to healthy control arteries ($p < 0.001$). Conversely, *CYP27B1* and *RUNX-2* mRNA was significantly increased by 94% and by 172% in CKD arteries, respectively ($p < 0.001$, $p < 0.0001$). *CYP24A1* mRNA levels in CKD arteries, compared to those in healthy arteries were not significantly different ($p = 0.20$) (*Figure 3.5, data provided by Dr Guerman Molostvov*).

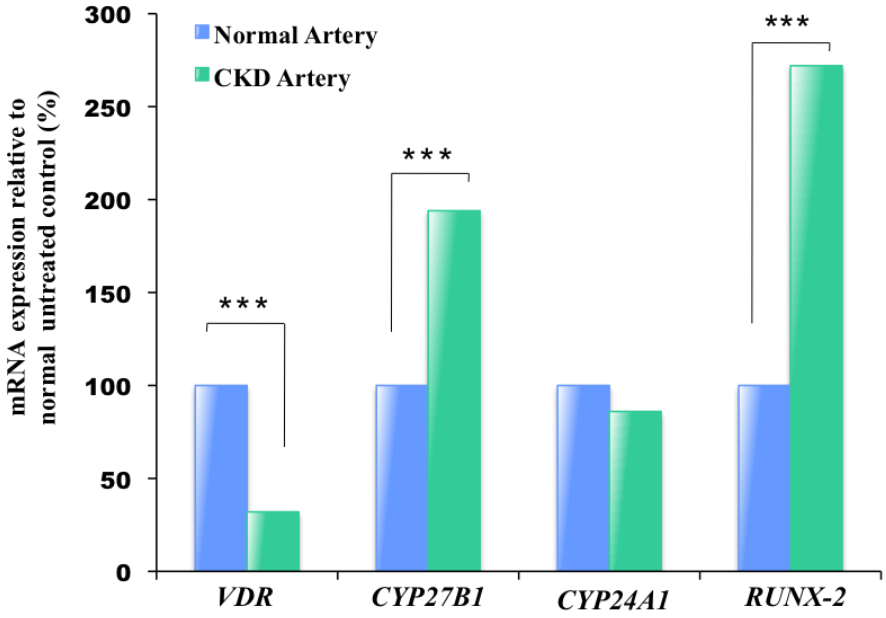


***Figure 3.5: Relative expression of Vitamin D Receptor (VDR), 24-Hydroxylase (CYP24A1), 1α-Hydroxylase (CYP27B1) and Runt-related Protein 2 (RUNX-2) mRNA in normal and Chronic Kidney Disease (CKD) arteries.*** *Results demonstrated by real-time RT-PCR. Values were corrected to 18S rRNA expression levels. *** $p < 0.001$, as calculated by one-way ANOVA; (n=11-17).*

### *3.2.2.2 Protein Expression of the Receptor and the Enzymes of the Vitamin D Hormonal System in HAoSMCs and Human Artery*

Arterial explant samples from a group of nine healthy individuals and nine CKD patients (all undergoing haemodialysis) were obtained with an informed consent. The

patient details including age, gender, blood pressure, hypertension, diabetes, CKD stage, renal diagnosis, blood haemoglobin, serum creatinine, serum calcium, phosphate and eGFR are outlined in Table 3.2.

***Table 3.2 Summary of Demographic and Clinical Data of Patients Included in Protein Expression Analyses (Western Blot).*** *Chronic Kidney Disease (CKD), Haemodialysis (H), Body Mass Index (BMI), Autosomal Dominant Polycystic Kidney Disease (ADPKD).*

| Patient Characteristics | Healthy | CKD | P |
|---|---|---|---|
| Age (median, range) | 55 (23-79) | 53 (26-64) | *NS* |
| Male (n,%) | 8 (80%) | 7 (70%) | *NS* |
| BMI (mean ± SD; kg/m$^2$) | 24.6 ± 4.6 | 23.7 ± 4.0 | *NS* |
| Nicotine (n,%) | 3 (30%) | 6 (60%) | |
| Blood Pressure (mean ± SD; mmHg) | | | |
| Systolic | 121 ± 10.6 | 137 ± 29.70 | *NS* |
| Diastolic | 66 ± 16.7 | 79 ± 13.7 | *NS* |
| Hypertension (n,%) | 1 (10%) | 9 (90%) | |
| Diabetes (n,%) | 0 | 0 | |
| CKD Stage (n) | | | |
| 5 | 0 | 1 | |
| 5D | 0 | 9 | |
| Renal diagnosis (n) | | | |
| ADPKD | - | 1 | |
| Glomerulonephritis | - | 5 | |
| Obstructive uropathy | - | 2 | |
| Other | - | 2 | |
| Laboratory (mean ± SD) | | | |
| Haemoglobin blood (g/dL) | 12.2 ± 1.1 | 11.6 ± 1.5 | *NS* |
| Creatinine serum (μM) | 98 ± 19.4 | 704 ± 121.9 | *<0.0001* |
| Calcium (mM) | 2.2 ± 0.22 | 2.1 ± 0.19 | *NS* |
| Phosphate (mM) | 1.2 ± 0.22 | 1.8 ± 0.52 | *<0.01* |
| eGFR (ml/min/1.73m$^2$) | 75 ± 12 | 7.2 ± 1.9 | *<0.0001* |

Western blot analyses were performed as described previously and expression was normalised to β-actin. On the following figures data are presented in a numbers of formats. A representative Western blots are shown and data are represented graphically. B for better visualisation, data were also presented in a form of a dot plot, where positioning from the mean was illustrated graphically for each individual

data point (mean was depicted by bars), for both groups. Data sets, in which data were non-normally distributed, were transformed by means of natural logarithm (ln) and then presented in a form of a dot plot.

Western blot analyses indicated that expression of the VDR, 1α-OHase and 24-OHase proteins was altered in CKD. VDR protein expression was not statistically different in CKD *(Figure 3.6).* 1α-OHase protein expression in CKD arteries was significantly reduced by 27% compared to the control group (*p=0.048*, as determined by an unpaired Student's t-test) *(Figure 3.7).* 24-OHase protein was not significantly changed (*p= 0.15* and *p = 0.12*, respectively) (*Figure 3.8*), a larger patient group may be needed to reduce the observed variability and confirm these findings. Interestingly, when relative 24-OHase protein were plotted as a function of relative 1α-OHase protein levels for all nine normal patients, a line showing almost a directly proportional relationship was observed ($R^2$=0.18) (*p < 0.0001*) *(Figure 3.9A).* This relationship appeared lost in CKD arteries, represented by almost a flat line ($R^2$=0.002) (*p < 0.0001) (Figure 3.9B).* This may suggests an increased 24-OHase catabolism in CKD, although this would have to be confirmed by measuring 24-OHase activity.

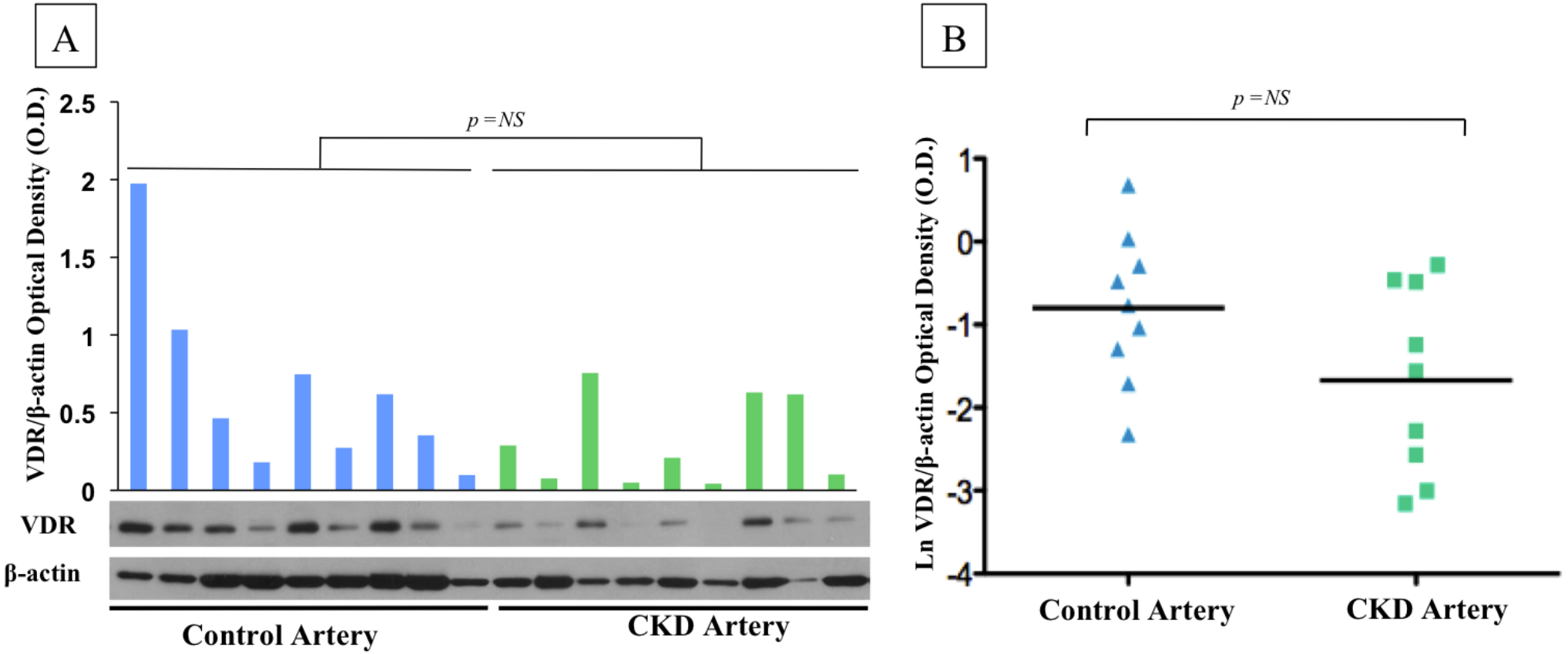


***Figure 3.6: The Vitamin D Receptor (VDR) protein expression in arteries from healthy individuals (Control Artery) and patients with advanced Chronic Kidney Disease (CKD).*** *(A) VDR protein expression in the arteries from patients with CKD was decreased (31%), compared to expression in control arteries, as demonstrated by Western blot analysis. (B) The same data presented in the form of a dot plot, where the distance from the mean (mean represented by a bar) is presented graphically for each individual data point; n=9 for each group. Reduced VDR protein expression in CKD compared to control artery was not statistically significant (NS), p=0.15, p value calculated by Student's t-test (data were normalised by logarithmic (ln) transformation prior to statistical analysis).*

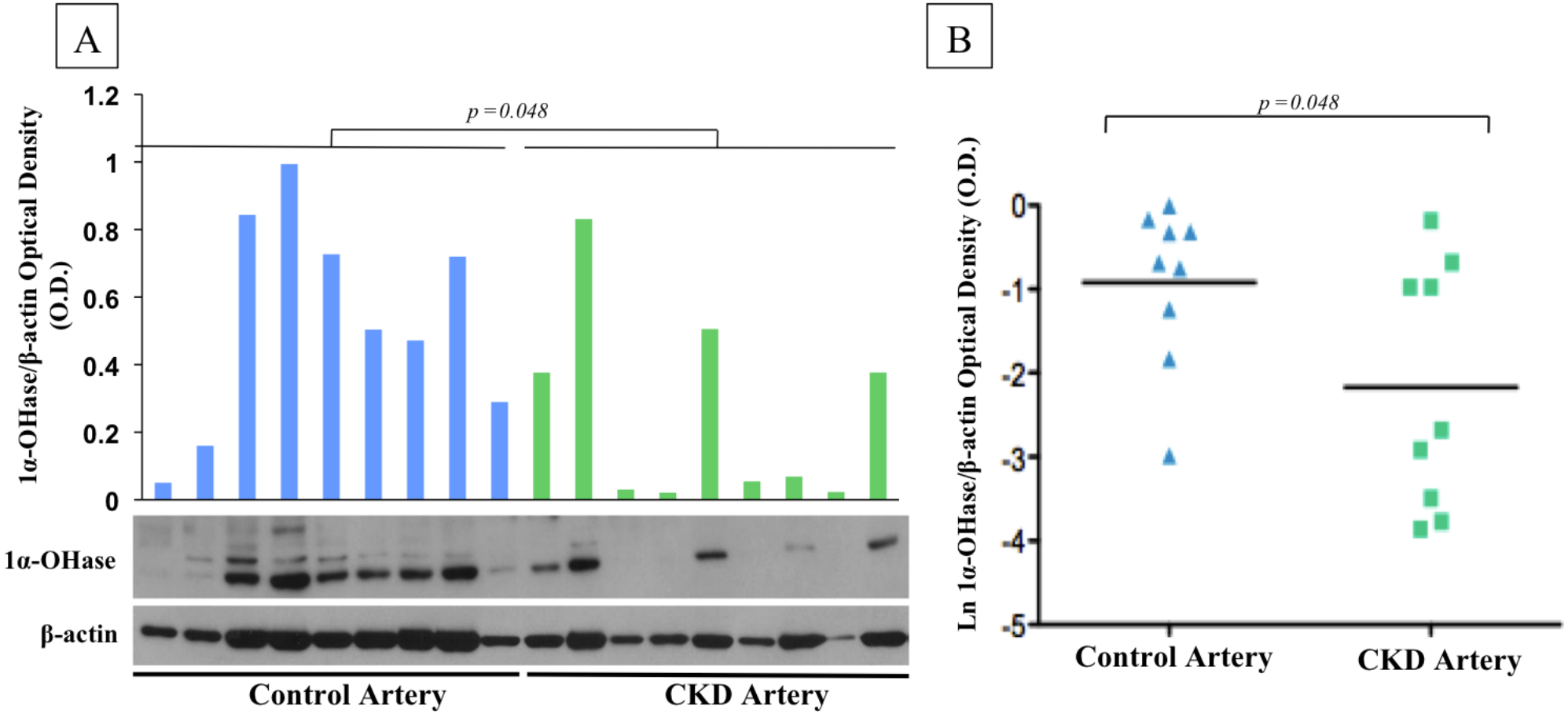


***Figure 3.7: The 1α-Hydroxylase (1α-OHase) protein expression in arteries of healthy individuals (Control Artery) and arteries of patients with advanced Chronic Kidney Disease (CKD).*** *(A) 1α-OHase protein expression is significantly decreased (27%) in the arteries from patients with CKD, compared to control arteries, as demonstrated by Western blot analysis. (B) The same data presented in the form of a dot plot, where the distance from the mean (mean depicted by a bar) is presented graphically for each individual data point, n=9 for each group, p=0.048, p value calculated by Student's t-test (data were normalised by logarithmic (ln) transformation prior to statistical analysis).*

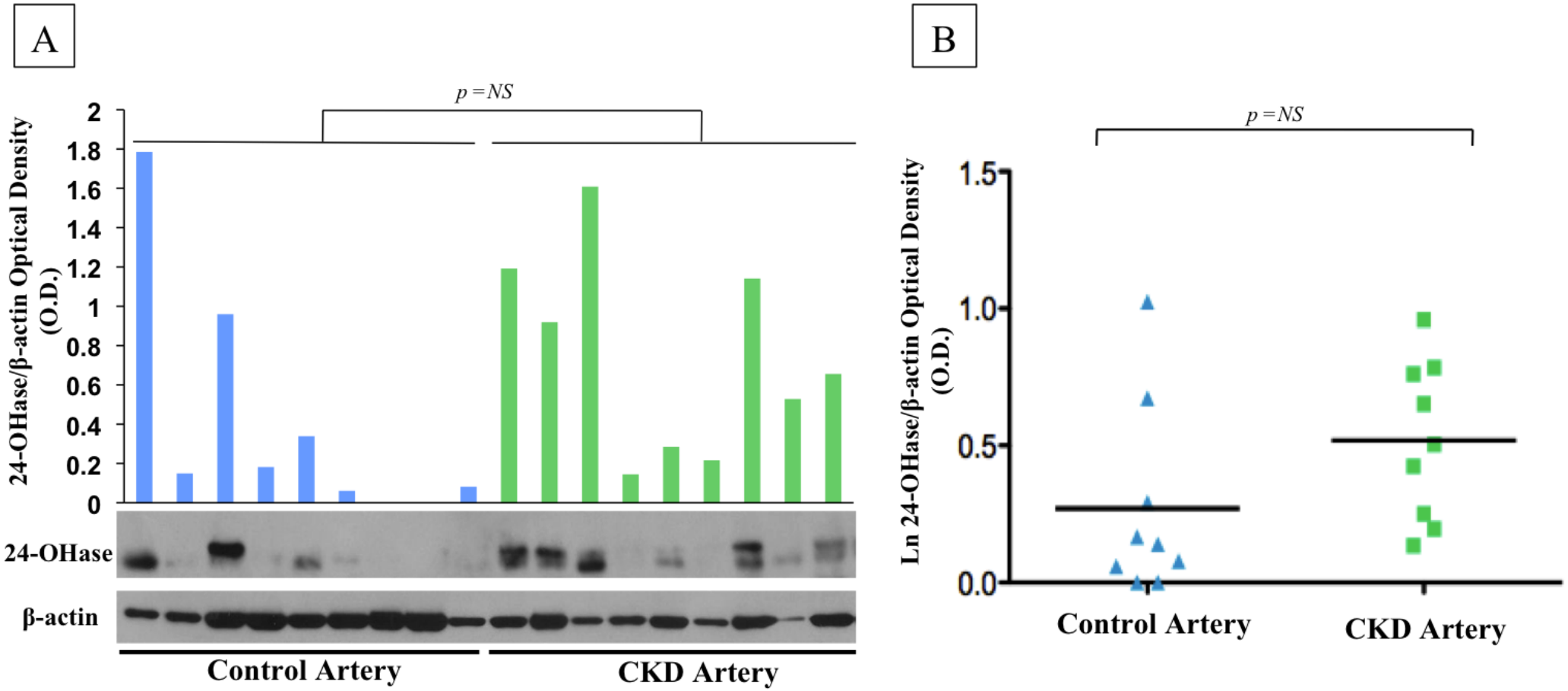


***Figure 3.8: The 24-Hydroxylase (24-OHase) protein expression in arteries from healthy individuals (Control Artery) and arteries from patients with advanced Chronic Kidney Disease (CKD)***. *(A) 24-OHase protein expression in the arteries from patients with advanced CKD was increased (49%), compared to expression in control arteries, as demonstrated by Western blot analysis. (B) The same data presented in the form of a dot plot, where distance from the mean (mean depicted by a bar) is presented graphically for each individual data point, n=9 for each group. Increased 24-OHase protein expression in CKD compared to control artery was not statistically significant (NS), p=0.12, p value calculated by Student's t-test (data were normalised by logarithmic (ln) transformation prior to statistical analysis).*

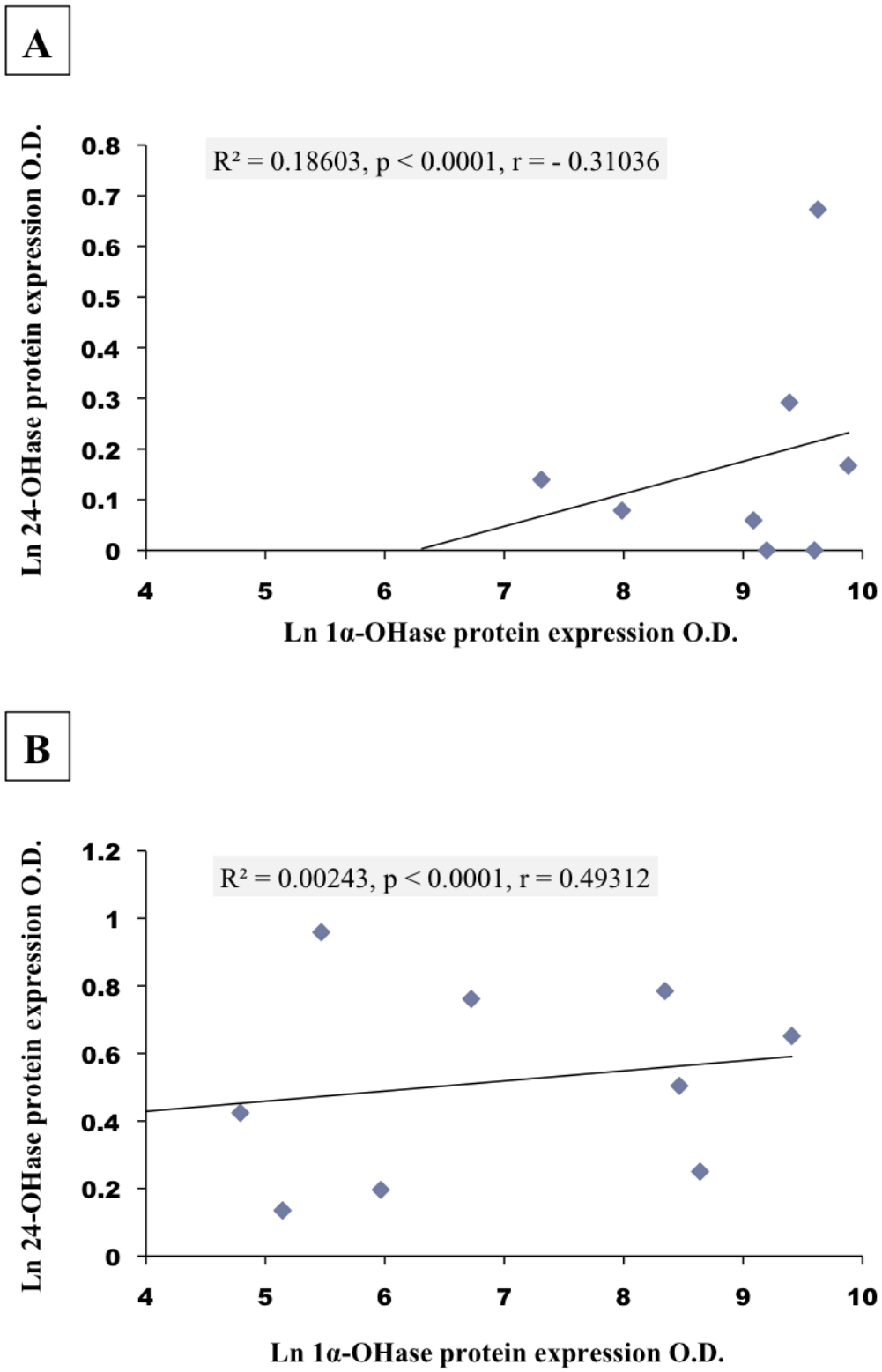


***Figure 3.9: Linear correlation between 24-Hydroxylase (24-OHase) protein level and 1α-Hydroxylase (1α-OHase) protein level, in healthy and Chronic Kidney Disease (CKD) arteries.*** *Values expressed as natural log Ln of optical density (O.D.), corrected by β-actin A) Normal arteries, negative correlation $R^2$=0.186, r=-0.31, p< 0.0001 (n=8) B) CKD arteries, positive correlation $R^2$=0.003, r=0.49 (n=9).*

Expression of RUNX-2, ALP and sclerostin in arteries, was used to indicate the stage of osteoblastic differentiation of VSMCs in the artery. High levels of α-actin were indicative of contractile, VSMC-like phenotype.

α-Actin *(Figure 3.10* , RUNX-2 *(Figure 3.11).* and ALP *(Figure 3.12)* protein levels were not significantly different between control and CKD group, as demonstrated by Western blot analyses. Protein expression of α-actin was fairly uniform across both groups, as indicated by similar means (0.63 and 0.60) *(Figure 3.10).*

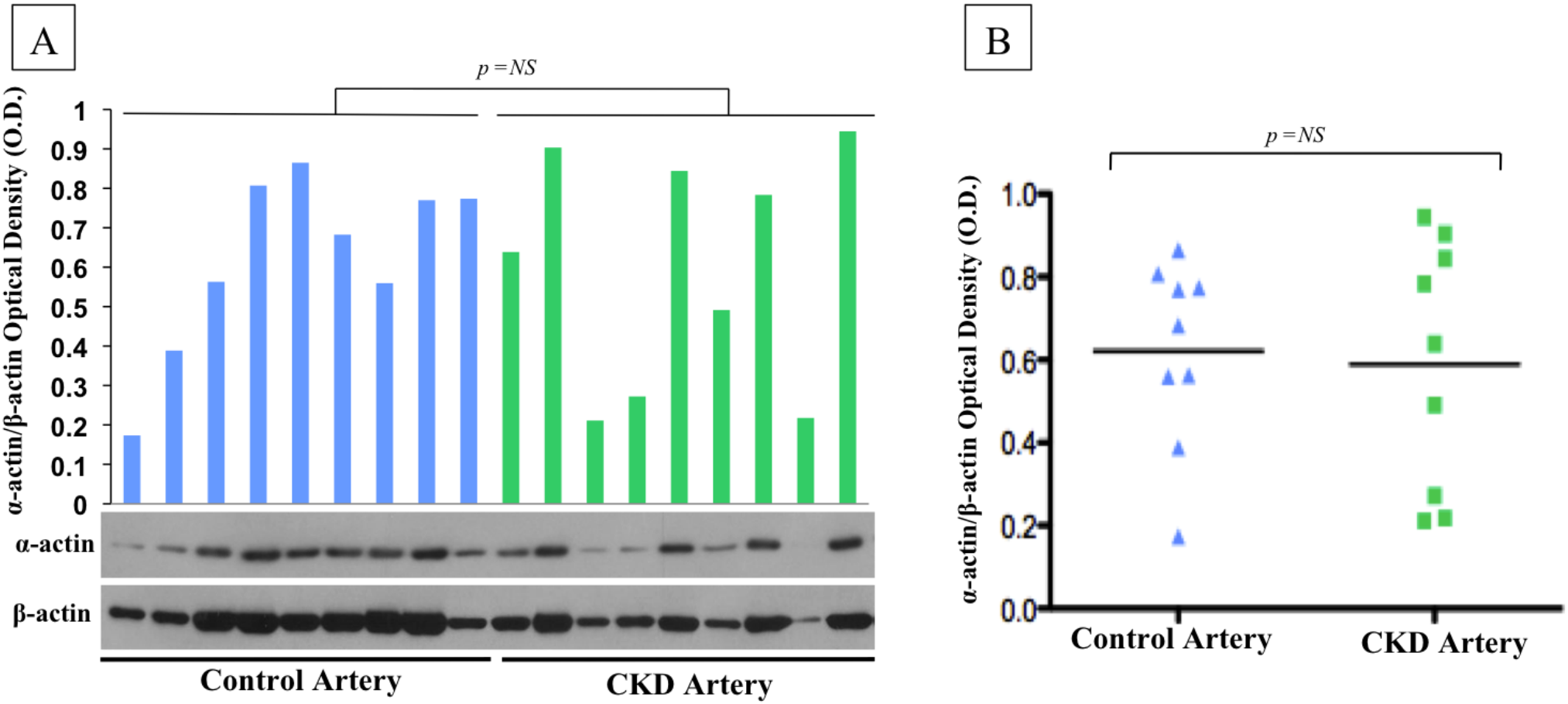


***Figure 3.10: The α-Actin protein expression in arteries from healthy individuals (Control Artery) and arteries from patients with advanced Chronic Kidney Disease (CKD)****. (A) α-Actin protein expression in the arteries from normal patients and those with advanced CKD appears to be fairly uniform, with no significant changes observed, as demonstrated by Western blot analysis. (B) The same data presented in the form of a dot plot, where distance from the mean (mean depicted by a bar) is presented graphically for each individual data point, n=9 for each group. Not statistically significant (NS), p=0.93, p value calculated by Student's t test.*

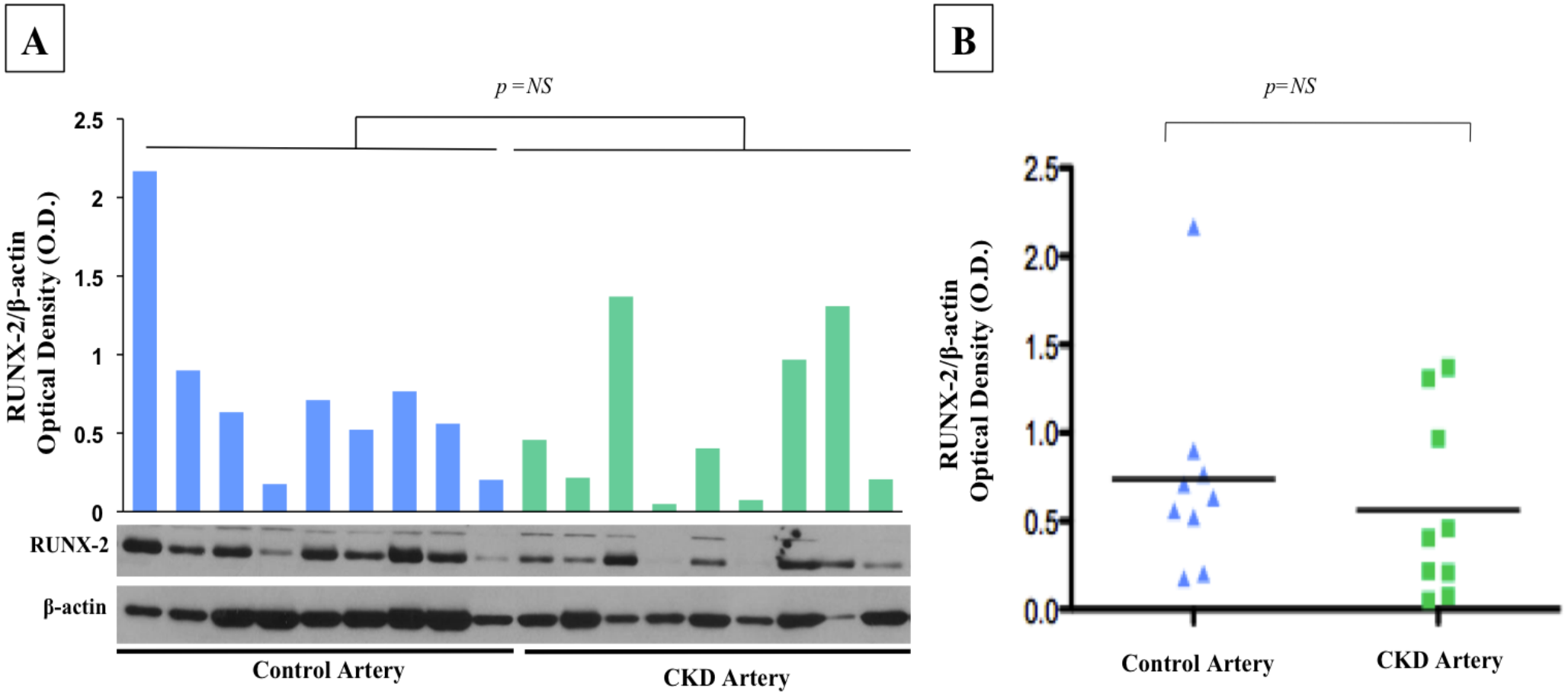


***Figure 3.11: The Runt-related transcription factor 2 (RUNX-2) expression in arteries from healthy individuals (Control Artery) and arteries from patients with advanced Chronic Kidney Disease (CKD)***. *(A) RUNX-2 protein expression in the arteries from normal patients appeared similar, if not slightly increased, compared to the expression in those with advanced CKD, as demonstrated by Western blot analysis. (B) The same data presented in the form of a dot plot, where distance from the mean (mean depicted by a bar) is presented graphically for each individual data point, n=9 for each group. Not statistically significant (NS) change, p=0.27, p value calculated by Student's t-test.*

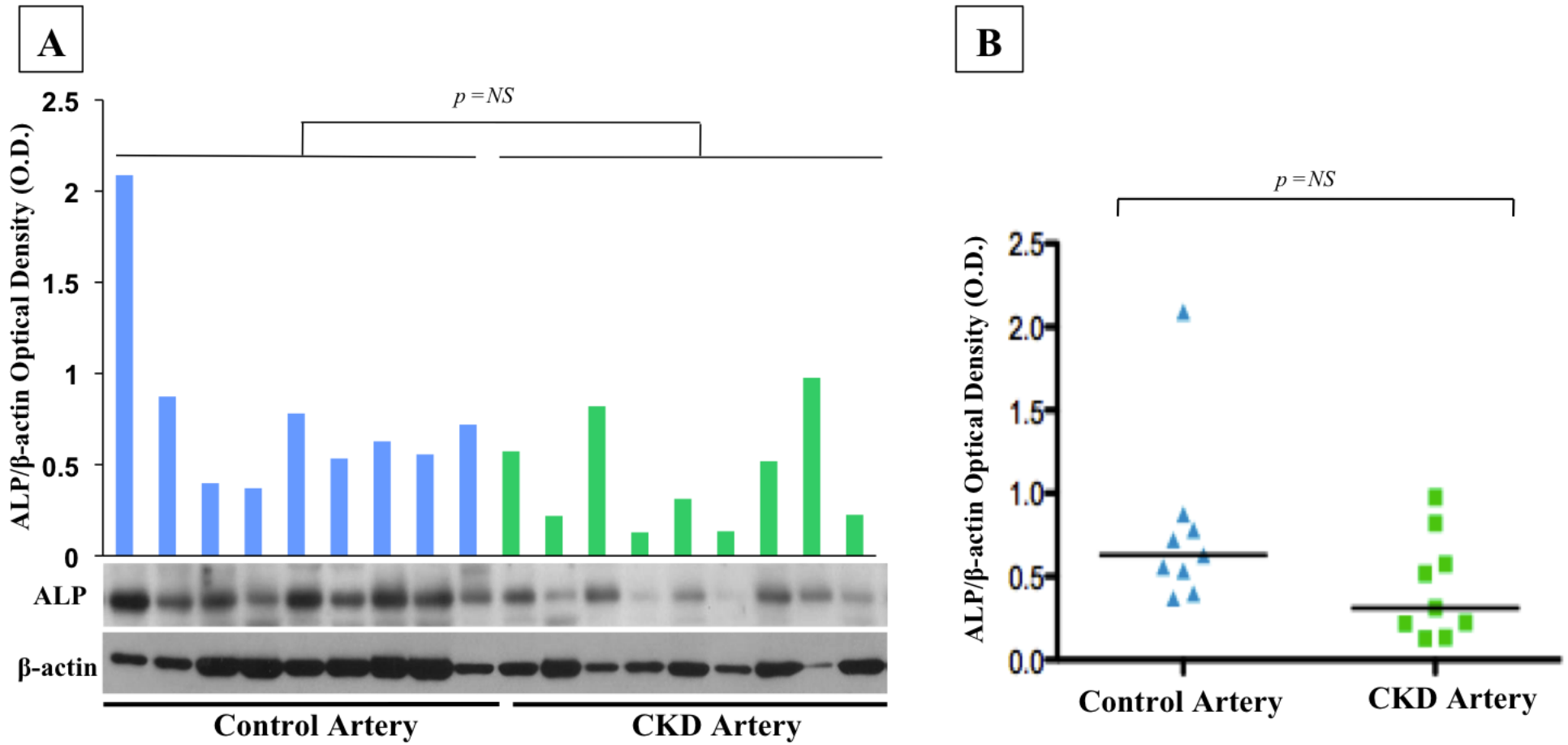


***Figure 3.12: The Alkaline Phosphatase (ALP) protein expression in arteries from healthy individuals (Control Artery) and arteries from patients with advanced Chronic Kidney Disease (CKD)***. *(A) ALP protein expression in the arteries from normal patients appeared to be elevated, as demonstrated by Western blot analysis. (B) The same data presented in the form of a dot plot, where distance from the mean (mean depicted by a bar) is presented graphically for each individual data point, n=9 for each group. Not statistically significant change (NS), p=0.06, p value calculated by Student's t test.*

Figure 3.13 is a summary of all arterial Western blot analyses to allow for easy characterization of the protein expression profile of an individual patient.

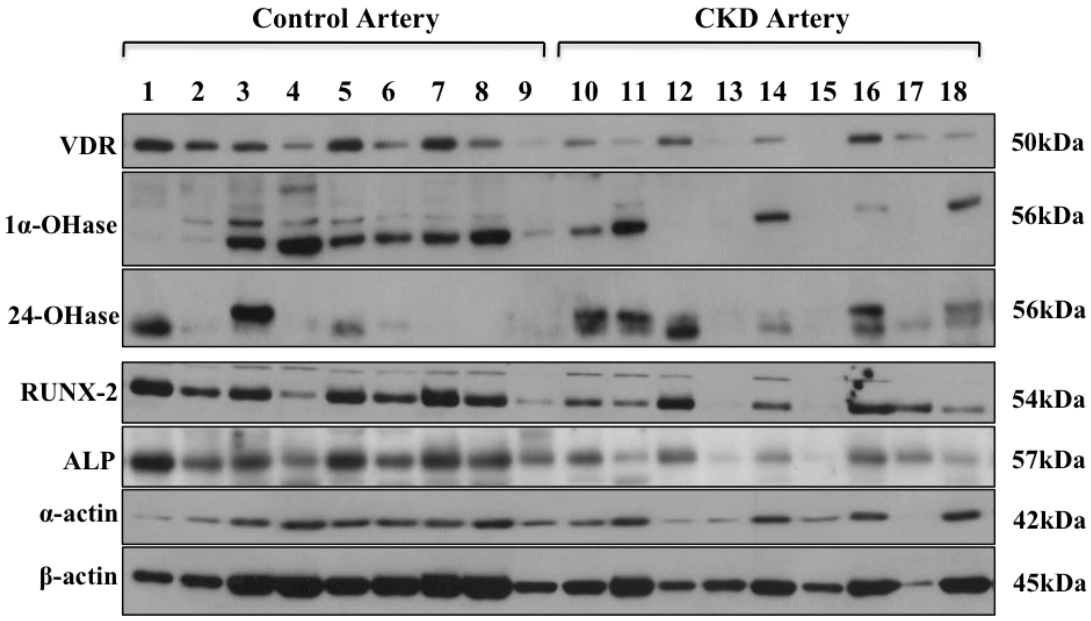


***Figure 3.13: Protein expression of the Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) in normal human artery and chronic kidney disease (CKD), artery together with the corresponding protein expression of the Runt-related transcription factor 2 (RUNX-2), Alkaline Phosphatase (ALP) and α-Actin.*** *Representative Western blots. β-actin was used as a loading control, numbers 1-17 indicate arteries from individual patients.*

### *3.2.2.3 Localisation of the VDR, 1α-OHase, 24-OHase and Bone-Specific Proteins in Human Artery using Immunohistochemistry*

To further analyse expression and localisation of the vitamin D signalling elements, sections of artery were examined by immunohistochemistry. 15 healthy and 15 CKD arteries were obtained as described in 2.1. Sections were prepared and stained for VDR, 1α-OHase, 24-OHase, as well as RUNX-2, ALP, sclerostin and calcium (Alizarin Red), as described in 2.4.

The intensity of the positive staining (brown for protein expression; red for calcium) of each individual section was assessed using a microscope. Random areas were

chosen on separate occasions and. Relative staining intensity was ranked from 0 (no staining) through 1 (very weak), 2 (medium) and 3 (strong), data were averaged and summarized in Table 3.1. Figures representative of staining for each of the proteins, in arteries from healthy individuals and from people with CKD are shown (*Figures 3.14-3.19).*

Immunochistochemical staining for VDR and 1α-OHase in CKD was weaker than the staining in normal arteries *(Figure 3.15 and 3.16).* This was consistent with the previous results from Western blot analyses *(Figure 3.5 and 3.6).* Again, consistent with my earlier findings, 24-OHase protein expression in CKD arteries appeared to be elevated, compared to healthy arteries *(Figure 3.17).* Calcification in all sections was assessed by Alizarin red (positive staining is red). No calcification was detected in any of the 15 healthy arteries. Of the 15 CKD arteries, moderate calcification was present in 3 and severe calcification was detected in another 3 arteries, the remaining 9 arteries did not appear to have any calcium deposits. Interestingly, it was observed that areas of calcification co-localised with the decreased expression of VDR *(Figure 3.13)* and 1α-OHase *(Figure 3.14)* and elevated expression of 24-OHase protein *(Figure 3.15)* in CKD, compared to healthy arteries. RUNX-2 protein was increased in CKD arteries, compared to expression in normal vessels *(Figure 3.17).* This finding was more convincing, than the data from Western blot analyses and consistent with the published findings (Moe *et al.* 2002, Tyson *et al.* 2003, Zhu *et al.* 2011). Similarly, low sclerostin expression seemed to be associated with high RUNX-2 protein levels or high calcium content in the medial layer *(Table 3.4 and Figure 3.19).* Immunohistochemistry results for ALP were similar to the data from Western blot analyses, where healthy arteries appeared to have higher levels of ALP, compared with healthy controls *(Figure 3.18).*

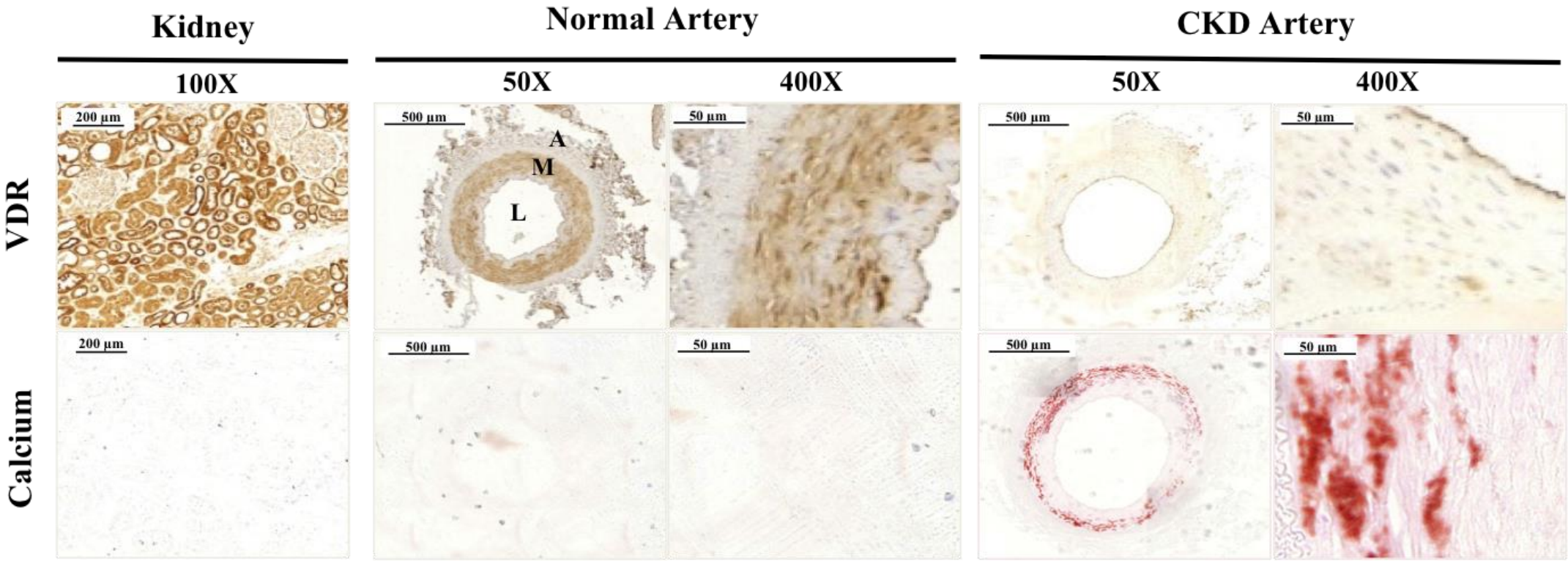


***Figure 3.14: The Vitamin D Receptor (VDR) protein expression and localisation in healthy (normal) human artery and in Chronic Kidney Disease (CKD) artery.*** *The same arterial sections stained with Alizarin Red for calcium. VDR positive staining (brown), calcium (red). VDR staining in the media (M) of CKD artery was weaker than the staining in the media of normal artery, as demonstrated by immunohistochemistry. Decreased VDR staining appeared to be linked to the presence of calcium deposits in the medial layer of the CKD artery. Magnifications: 50X, 100X, 400X. L-lumen, A-adventitia. Kidney cortex was used as positive control.*

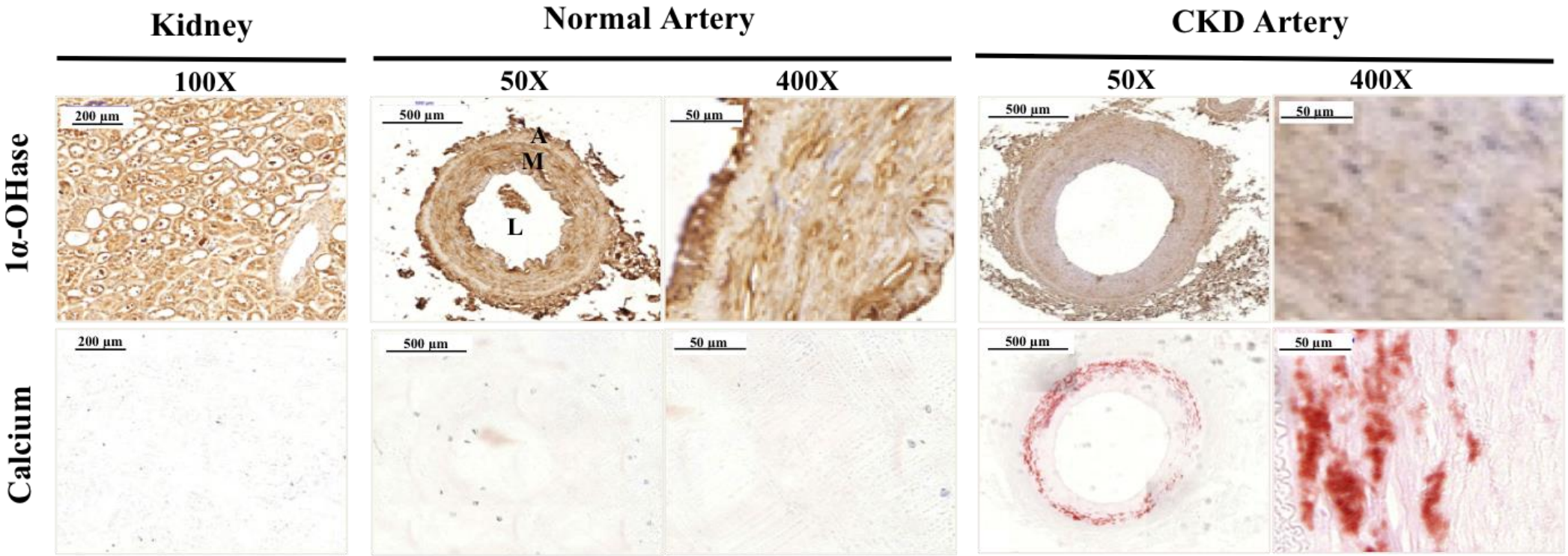


***Figure 3.15: The 1α-Hydroxylase (1α-OHase) protein expression and localisation in healthy (normal) human artery and in Chronic Kidney Disease (CKD) artery.*** *The same arterial sections stained with Alizarin Red for calcium. 1α-OHase positive staining (brown), calcium (red). 1α-hydroxylase staining in the media (M) of CKD artery was weaker than the staining in the media of normal artery, as demonstrated by immunohistochemistry. Decreased 1α-OHase staining appeared to be linked to the presence of calcium deposits in the medial layer of CKD artery. Magnifications: 50X, 100X, 400X. L-lumen, A-adventitia. Kidney cortex was used as positive control.*

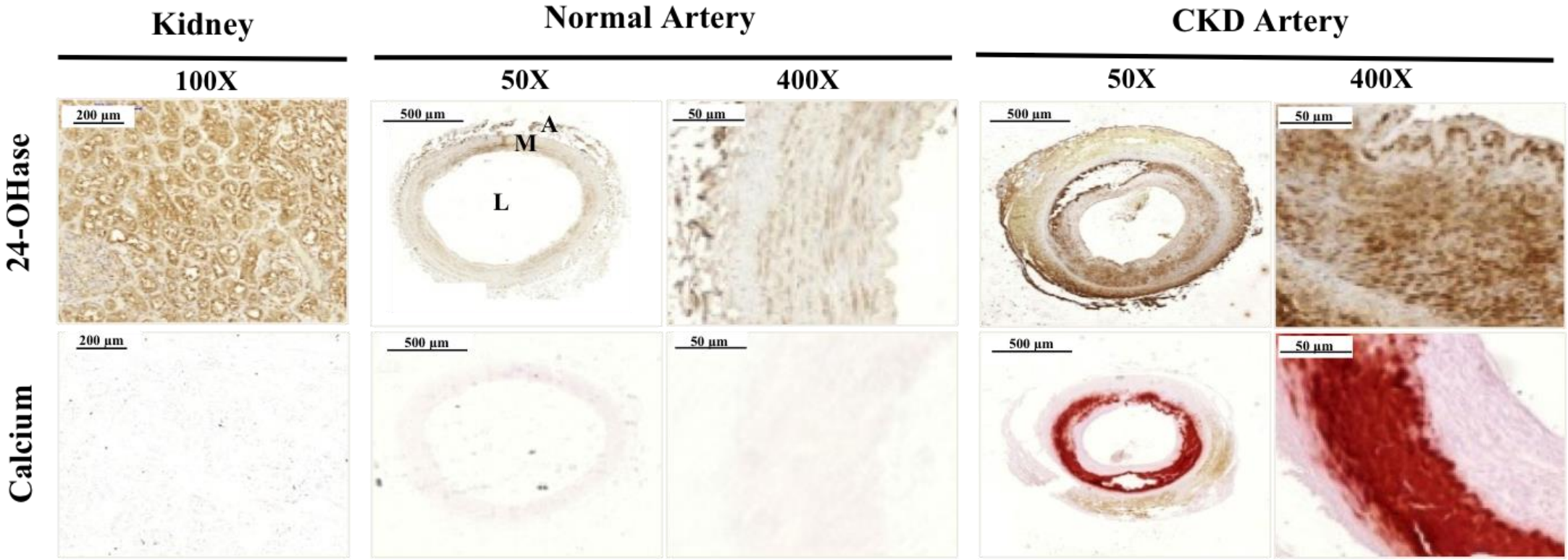


***Figure 3.16: The 24-Hydroxylase (24-OHase) protein expression and localisation in healthy (normal) human artery and in Chronic Kidney Disease (CKD) artery.*** *The same arterial sections stained with Alizarin Red for calcium. 24-OHase positive staining (brown), calcium (red). 24-OHase staining in the media (M) of CKD artery was stronger than the staining in the media of normal artery, as demonstrated by immunohistochemistry. Increased 24-OHase staining appeared to be linked to the presence of calcium deposits in the medial layer of CKD artery. Magnifications: 50X, 100X, 400X. L-lumen, A-adventitia. Kidney cortex was used as positive control.*

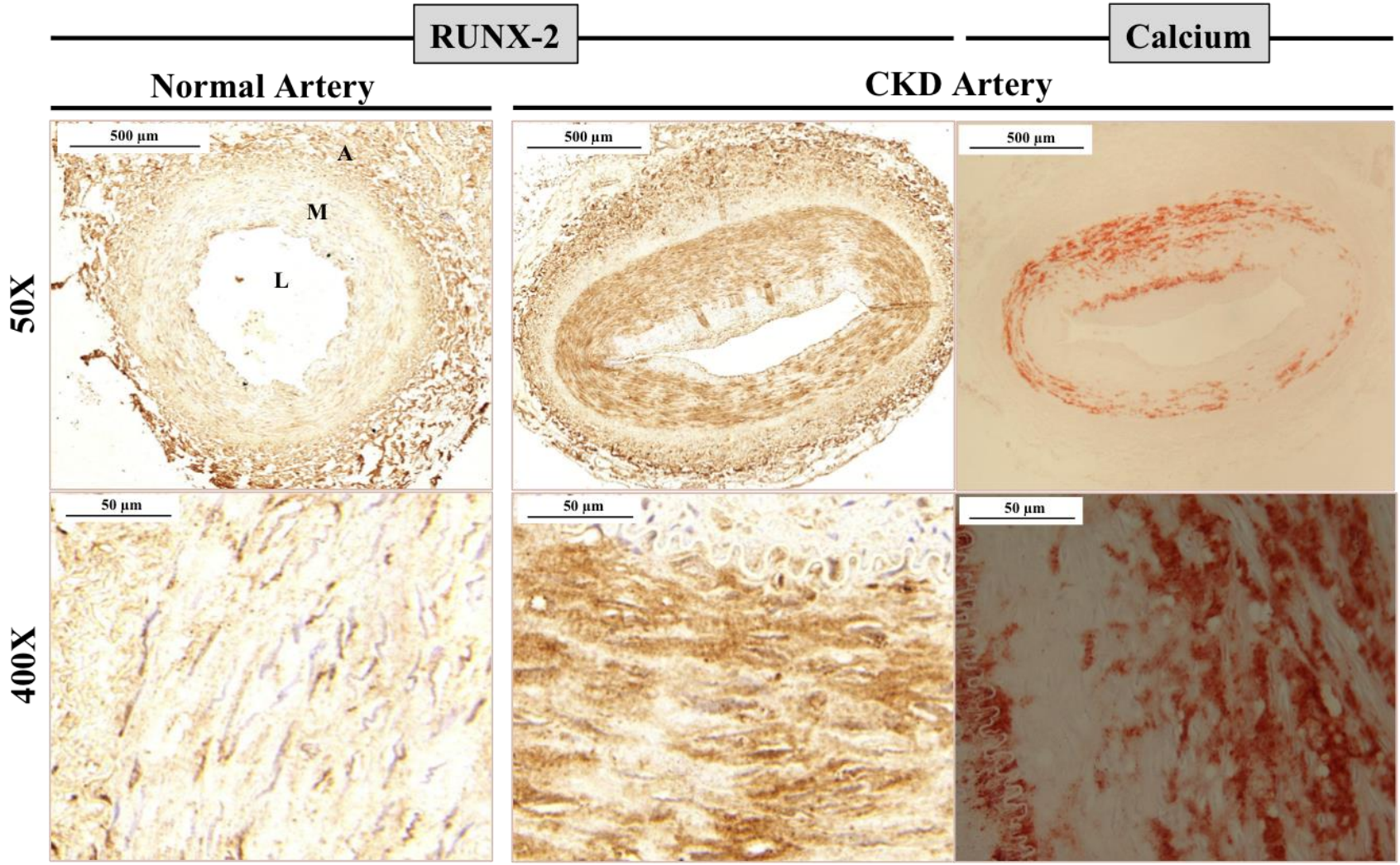


***Figure 3.17: The Runt-related Transcription Factor 2 (RUNX-2) protein expression and localisation in healthy (normal) artery and in Chronic Kidney Disease (CKD) artery****. The same arterial sections stained with Alizarin Red for calcium. RUNX-2 positive staining (brown), calcium (red). RUNX-2 staining in the media (M) of CKD artery was stronger than the staining in the media of normal artery, as demonstrated by immunohistochemistry. Increased RUNX-2 staining appeared to be linked to the presence of calcium deposits in the medial layer of the CKD artery. Magnifications: 50X, 400X. L-lumen, A-adventitia.*

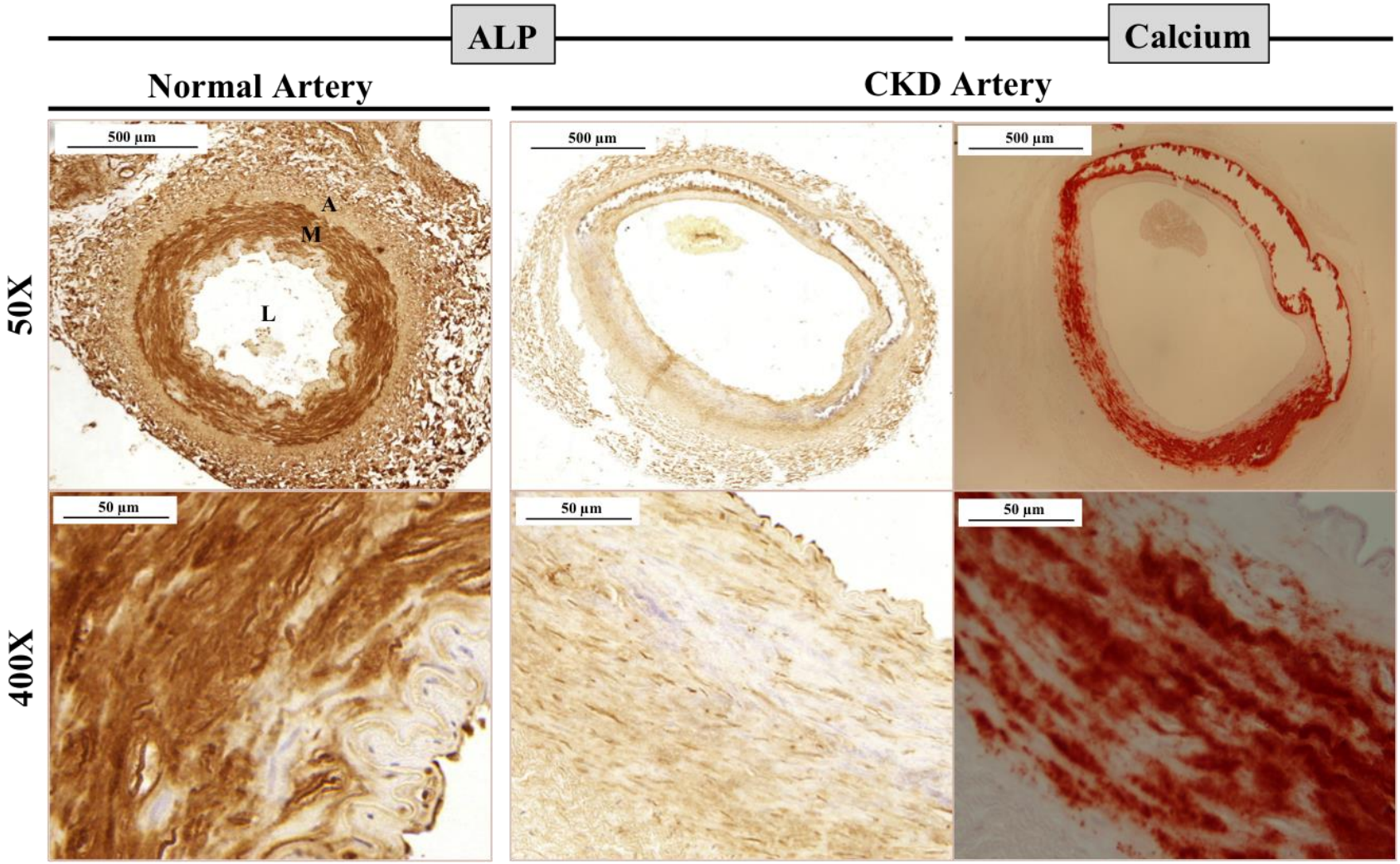


***Figure 3.18: The Alkaline Phosphatase (ALP) protein expression and localisation in healthy (normal) human artery and in Chronic Kidney Disease (CKD) artery****. The same arterial sections stained with Alizarin Red for calcium. ALP positive staining (brown), calcium (red). RUNX-2 staining in the media (M) of normal artery was stronger than the staining in the media of a CKD artery, as demonstrated by immunohistochemistry. ALP staining (individual cells) appeared to co-localise with calcium in the medial layer of CKD artery. Magnifications: 50X, 400X. L-lumen, A-adventitia*

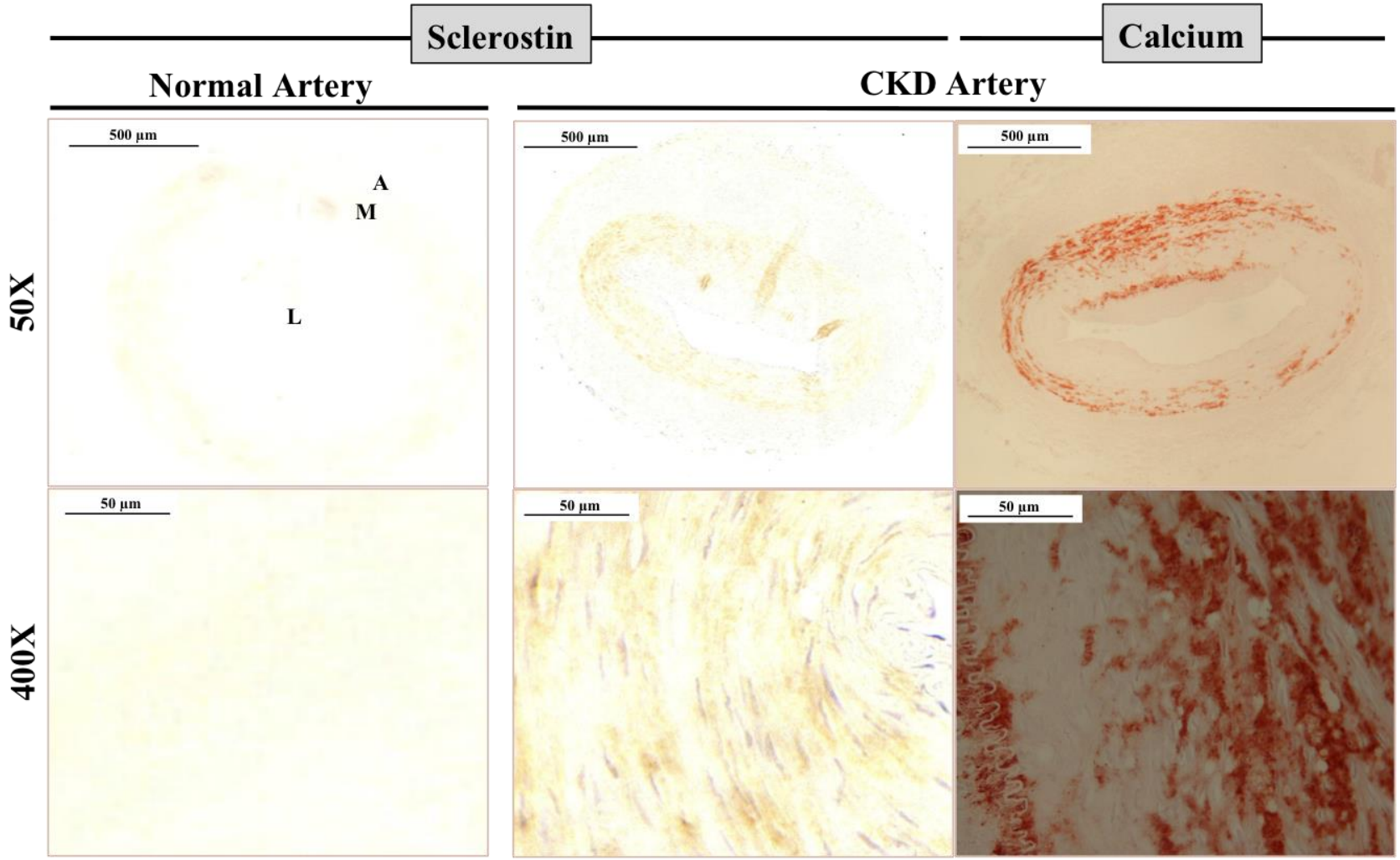


***Figure 3.19: The Sclerostin protein expression and localisation in healthy (normal) human artery and in Chronic Kidney Disease (CKD) artery***. *The same arterial sections stained with Alizarin Red for calcium. Sclerostin positive staining (brown), calcium (red). RUNX-2 staining in the media (M) of normal artery was stronger than the staining in the media of a CKD artery, as demonstrated by immunohistochemistry. Sclerostin staining (individual cells) appeared to co-localise with calcium in the medial layer of CKD artery. Magnifications: 50X, 400X. L-lumen, A-adventitia.*

***Table 3.3: Estimation of the Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase), 24-Hydroxylase (24-OHase), Runt Related Transcription Factor 2 (RUNX-2), Alkaline Phosphatase (ALP), Sclerostin and calcium staining intensity in the arterial sections from healthy and Chronic Kidney Disease (CKD) patients.*** *Staining intensity (% intensity of brownness in the medial layer) was assessed by eye. Three random areas were chosen on 3 separate occasions (n=15 healthy, n=15 CKD) Staining was scored subjectively as: 0 (no staining) through 1 (very weak staining), 2 (medium staining) and 3 (strong staining). Data represent median scores from 15 sections. Presence of calcification scores - in red and highlighted. Mode, range and average were calculated.*

**Healthy Arteries**

| *Specimen* | *VDR* | *1α-OHase* | *24-OHase* | *RUNX-2* | *ALP* | *Sclerostin* | *Calcium* |
|---|---|---|---|---|---|---|---|
| *1* | 3 | 2 | 3 | 1 | 3 | 2 | 0 |
| *2* | 3 | 2 | 3 | 0 | 2 | 0 | 0 |
| *3* | 3 | 2 | 3 | 1 | 3 | 0 | 0 |
| *4* | 2 | 2 | 1 | 0 | 1 | 0 | 0 |
| *5* | 2 | 3 | 1 | 0 | 3 | 0 | 0 |
| *6* | 1 | 2 | 3 | 0 | 1 | 0 | 0 |
| *7* | 2 | 2 | 3 | 2 | 3 | 0 | 0 |
| *8* | 2 | 3 | 3 | 2 | 2 | 2 | 0 |
| *9* | 2 | 3 | 1 | 2 | 3 | 2 | 0 |
| *10* | 1 | 2 | 3 | 0 | 1 | 0 | 0 |
| *11* | 1 | 3 | 3 | 0 | 2 | 0 | 0 |
| *12* | 3 | 2 | 3 | 0 | 2 | 0 | 0 |
| *13* | 1 | 3 | 3 | 0 | 3 | 0 | 0 |
| *14* | 1 | 1 | 2 | 0 | 3 | 0 | 0 |
| *15* | 1 | 3 | 1 | 0 | 3 | 0 | 0 |
| MODE | 1 | 2 | 3 | 0 | 3 | 0 | 0 |
| RANGE | 2 | 2 | 2 | 2 | 2 | 2 | 0 |
| AVERAGE | 1.87 | 2.33 | 3.75 | 0.53 | 2.33 | 0.4 | 0 |

**CKD Arteries**

| *Specimen* | *VDR* | *1α-OHase* | *24-OHase* | *RUNX-2* | *ALP* | *Sclerostin* | *Calcium* |
|---|---|---|---|---|---|---|---|
| *1* | 3 | 1 | 2 | 0 | 1 | 0 | 0 |
| *2* | 2 | 2 | 0 | 0 | 1 | 0 | 0 |
| *3* | 3 | 3 | 1 | 3 | 2 | 0 | 0 |
| *4* | 3 | 2 | 2 | 0 | 1 | 0 | 0 |
| *5* | 3 | 3 | 2 | 3 | 2 | 2 | 2 |
| *6* | 2 | 2 | 1 | 3 | 2 | 1 | 3 |
| *7* | 3 | 2 | 1 | 0 | 1 | 0 | 0 |
| *8* | 1 | 2 | 2 | 3 | 0 | 0 | 3 |
| *9* | 1 | 2 | 1 | 3 | 2 | 2 | 2 |
| *10* | 2 | 2 | 2 | 0 | 0 | 0 | 0 |
| *11* | 3 | 2 | 3 | 3 | 1 | 1 | 3 |
| *12* | 2 | 1 | 1 | 0 | 0 | 0 | 0 |
| *13* | 1 | 1 | 3 | 0 | 1 | 0 | 0 |
| *14* | 3 | 3 | 3 | 0 | 2 | 0 | 1 |
| *15* | 1 | 2 | 3 | 2 | 2 | 1 | 0 |
| MODE | 3 | 2 | 2 | 0 | 1 | 0 | 0 |
| RANGE | 2 | 2 | 2 | 3 | 2 | 2 | 3 |
| AVERAGE | 2.2 | 2 | 3.19 | 1.33 | 1.2 | 0.47 | 0.93 |

## 3.3 Discussion

Current research in the area of vascular calcification and vitamin D has focused primarily around endocrine hormonal system, the importance of which, categorically, cannot be underestimated. However, vitamin D metabolites, particularly $1,25(OH)_2D_3$, are able to act on a range of not only systemic but also local processes in vascular cells. The recent result of PRIMO study (Thadhani 2011), which failed to show the long term effect of the paricalcitol administration on cardiac structure and function in cardiac haemodialysis patients with left ventricular hypertrophy confirmed the need for more in-depth understanding of the complex interrelationship between vitamin D system and vasculature.

This study demonstrated presence of VDR mRNA and protein in human VSMC and in human artery. RT-PCR analyses showed that *VDR* PCR product was detectable in VSMCS and that it was similar in size to the transcript detected in HKC-8 cells. The method for VDR protein detection was carefully validated and is described in Chapter 2. Presence of VDR protein in VSMCs and human artery was conformed where a dublet of bands was detected (approximately 50 kDa and 52 kDa). 48 kDa protein has been previously detected in human intestinal semian virus (CV-1) cells engineered to over express VDR, whereas 50 kDa protein has been detected in the membrane fraction of the same cells (Bula *et al.* 2005). Literature suggests that the 48 kDa protein is likely to be a product of transcription starting at the first ATG codon in exon 2, methionine 4 (Bula *et al.* 2005). It has also been suggested that both 48 and 52 kDa may be other nuclear matrix proteins with C-terminal homologous to that of VDR (Nangia *et al.* 1998). The nature of higher molecular mass protein cross-reacting with VDR antibody, which has been observed by others is not known (Bula *et al.* 2005, Krishnan and Feldman 1992, Kumar *et al.* 1994), it may also be a

different VDR isoform or a result of posttranslational modifications, such as phosphorylation or glycosylation.

Despite multiple publications demonstrating the presence of VDR in smooth muscle tissues other groups strongly argue that the receptor is not there (Wang Y. and DeLuca 2011). The primary argument dismissing the validity of previous research is the lack of specificity of antibodies used in the previous studies (allegedly non-specific C-20 and 9A7, as opposed to specific D-6). As it is harder to disagree with research showing variety of effects that 1,25$(OH)_2$D exerts on SMCs, it is argued by the authors that these effects are indirect. Further, the same authors state that according to the unpublished results, mRNA expression levels in intestinal SMCs are extremely low, which may explain why VDR protein expression is negligible to non-existent.

Further, mRNA and protein expression of two essential enzymes of the vitamin D system, 1α-OHase and 24-OHase (the latter for the first time) was shown by us in VSMCs and human artery, which suggests that vascular VDR-1,25$(OH)_2$D exerts its actions through the genomic pathway. 24-OHase protein of approximately 56 kDa, the same size as detected by us has been previously shown to be present in kidney proximal tubule cells, breast cells, keratinocytes and other tissues (more on 24-OHase expression and regulation see chapter 1.3.3.3). Interestingly, in malignant breast tissues a band of 40 kDa is also present and it may be a result of alternative splicing of *CYP24A1* mRNA (Fischer *et al.* 2009). In our study no isoforms of 24-OHase were detected neither in VSMCs, healthy nor in CKD arteries suggesting that alternatively spliced *CYP24A1* mRNA is not necessarily an indication of a disease process *per se*, but may be more specifically associated with cancerous processes. First report of *CYP27B1* mRNA in human VSMC was by Somjen almost a decade

ago (Somjen *et al.* 2005), however to date there have been no reports demonstrating the expression of 1α-OHase protein in human VSMCs.

Importantly, the results from this study proved that there are differences in expression of the vitamin D system elements, in mRNAs and proteins between healthy and CKD arteries. The altered expression of both *VDR* mRNA (decreased) and *CYP27B1* mRNA (increased) was consistent with previous observations by others. Altered expression of renal VDR, 1α-OHase and 24-OHase in CKD has been previously shown both in humans and rats and has been addressed in Chapter 1 (Cozzolino *et al.* 2006, Dusso and Rodriguez 2012, Helvig *et al.* 2010). According to real-time RT-PCR analyses, *CYP27B1* mRNA levels were significantly higher in CKD arteries, by 94% compared to those in normal arteries. This finding was not reflected on the protein level, as according to Western blot analysis, 1α-OHase protein in CKD arteries was down by 27% compared to normal controls. This is a very interesting finding, which potentially could be explained by observations made by others in THP1 (human acute monocytic leukemia cell line) and human monocytes. It appears that monocytic baseline *CYP27B1* mRNA expression is increased in uraemia, possibly reflecting the low-grade inflammation present in CKD (Viaene *et al.* 2012). Interestingly, when cells were stimulated with pro-inflammatory cytokines (IL-6 or TNF-α or IL-1) in presence of uraemic serum, the induction of *CYP27B1* mRNA was impaired. Further, uraemic toxins such as indoxyl sulphate or p-cresyl sulphate were shown not to have any impact on *CYP27B1* levels. Taken together these findings may explain the high *CYP27B1* mRNA levels in CKD arteries and low 1α-OHase protein levels. Further, despite no interference with mRNA, uraemic toxins may interfere and ultimately reduce 1α-OHase protein translation. Further studies are needed to confirm this theory. Another potential explanation may be presence of small non-coding RNAs - micro RNA (miRNA)

species, which are responsible for the regulation of up to 30% of human genes, with capabilities to influence almost all cellular pathways (Sioud and Cekaite 2010). Recent evidence from studies on HKC-8 and THP-1 monocytic cells confirmed the existence of regulatory noncoding *CYP27B1* splice variants, which may have great impact on 1,25$(OH)_2$D synthesis in normal physiology (Wu *et al.* 2007). miRNAs are known to be dysregulated in cancer (Sioud and Cekaite 2010), it is therefore not unreasonable to speculate that CKD may also be a state of altered expression of certain miRNAs.

Decrease in *VDR* mRNA and protein in CKD arteries, although not significant (protein), may suggest that regulation of 1α-OHase may not only be exerted via a downstream action of VDR, but that it may be strongly dependent on the levels of 24-OHase, which in turn, may be regulated by some other indirect mechanism. In CKD arteries, levels of *CYP24A1* mRNA were similar to those in normal arteries, whereas levels of *CYP27B1* mRNA were significantly increased. Conversely, in CKD levels of 24-OHase protein, albeit non-significantly, were increased, as opposed to significantly reduced 1α-OHase protein levels. The question that remains to be answered is what exactly causes this dramatic shift post-transcription or pre/during translation.

Overall, only four out of 15 examined arteries were calcified (one was calcified moderately and there was no apparent calcification in the remaining 9, as indicated by alizarin red staining). Similar observations have been made in the past (Moe *et al.* 2002), where less than a half of the arteries from ESRD patients undergoing renal transplantation were calcified (out of 39 vessels, 12 were calcified and 27 showed no evidence of calcification). Interestingly, in this study, in the four severely calcified sections, the immunoreactivity for 24-OHase was very strong in two vessels and very

weak in the other two. Consequently, the expression of 1α-OHase in the severely calcified vessels followed the exact opposite pattern, suggesting that neither a decrease in 1α-OHase nor an increase in 24-OHase protein is a single determinant of a medial calcification. The VDR immunoreactivity in severely calcified arteries was consistently decreased compared with non-calcified arteries from patients undergoing haemodialysis. This may suggest that the VDR signalling is hampered in calcified vessel, but not the reciprocal regulation of 1α-OHase and 24-OHase. Further, the differences in expression of vitamin D system elements in calcified arteries may be attributed to status of $1,25(OH)_2D$ of an individual patient as well as the plausible VDRA treatment they might have been undergoing, also age of a patient may have an impact.

Western analyses of early (ALP, RUNX-2) and late osteoblastic markers (sclerostin) in CKD and the control group have not shown any significant sign in phenotypic transdifferentiation of VSMCs. Interestingly, it appeared that ALP protein levels were higher in healthy than in CKD arteries. This is in conflict with previous reports, which suggested elevated ALP in serum of CKD patients (Shantouf *et al.* 2009) and in calcified CKD arteries (Moe *et al.* 2002). Perhaps, if the test group was larger, clearer association would become more apparent. On the other hand, the immunohistochemistry studies showed an increase in RUNX-2 staining in CKD group (6 out of 15 very strong staining in CKD, as opposed to 5 out of 15 with weak to moderate staining in normal arteries). The staining appeared highest in arteries, which stained very weakly with alizarin red, confirming that increase in RUNX-2 occurs at an early stage of osteoblastic transdifferentiation. On the basis of recently published findings showing contradictory result (Moe *et al.* 2002, Tyson *et al.* 2003, Zhu *et al.* 2011) – perhaps increasing the patient number would decrease the variability ultimately impacting the result.

More evidence in the following chapters shows direct involvement of VDR in vitamin D system regulation within the VSMCs. Direct involvement of VDR in regulation of VSMC function was demonstrated few years ago through performing a DNA microarray study in human coronary artery SMCs (Wu-Wong *et al.* 2007b). *VDR* DNA was not only shown to be expressed at high levels, but also to be significantly upregulated (together with *CYP24A1*) as a result of $1,25(OH)_2D_3$ treatment. To date, the enzymatically functional 1α-OHase system in VSMCs was characterised, however the context of VDR and 24-OHase involvement was not addressed (Somjen *et al.* 2005). Due to the lack of published data in the area of vascular vitamin D system regulation, it is still not known whether VDR-24-OHase-1α-OHase feedback regulation is principally the same as described in kidney (Bland *et al.* 1999, Bland *et al.* 2001, Zehnder and Hewison 1999, Zehnder *et al.* 1999).

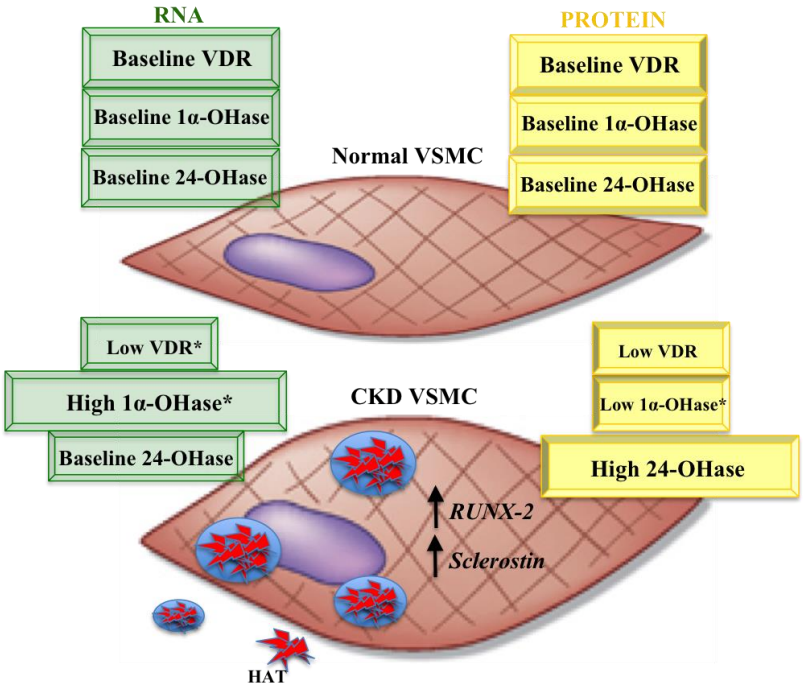


***Figure 3.20: Schematic Representation of the Changes in the Expression of Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase), 24-Hydroxylase (24-OHase) mRNA and Protein in Normal Vascular Smooth Muscle Cell (VSMC) and in Chronic Kidney Disease (CKD) VSMC, with accompanying osteoblastic changes.*** *Runt related Transcription Factor (RUNX-2), Hydroxyapatite crystals* $Ca_{10}(PO_4)_6(OH)_2$ *(HAT).*

Overall, 1α-OHase activity studies are needed to understand how relevant the observed 1α-OHase-24-OHase-VDR disconnection in CKD really is. On the basis of previous research, it is not unreasonable to assume that local vitamin D system in artery may be regulated similarly to the one described in human monocytes, i.e. by stimuli such as calcium, phosphate, proinflammatory cytokines or other important factors in CKD, such as FGF-23 or Klotho. Another important stimulus may be $1,25(OH)_2D$ itself and therefore the content of the next chapter has been dedicated to elucidation of that aspect entirely.

To summarise, the expression of 24-OHase protein and mRNA in human vasculature and primary cultures of HAoSMCs was demonstrated for the first time. Further, presence of VDR and 1α-OHase mRNA and protein was shown, which suggested a role for a local, arterial VDR activation. Also, the expression of 1α-OHase, 24-OHase and VDR protein and mRNA in normal and CKD arteries was compared. This led to the discovery that expression of 1α-OHase protein was decreased in CKD arteries compared to control, whereas VDR and 24-OHase protein expression was not statistically changed. Altered expression of the aforementioned elements in CKD arteries appeared to co-localise with medial calcification and increased levels of RUNX-2 and sclerostin.

# Chapter 4

# Regulation of the Vascular Vitamin D System by $1,25(OH)_2D_3$

## 4.1 Background

The importance of selective vascular VDR activation can be appreciated by understanding of the differences between the endocrine and an auto/paracrine vitamin D system. Treatment of vitamin D deficient patients with non-selective $1,25(OH)_2D_3$ as discussed in general introduction, leads to the correction of $1,25(OH)_2D_3$, where $1,25(OH)_2D_3$ acts systemically on intestinal calcium and phosphate absorption, bone metabolism, renal calcium absorption and parathyroid function. Evidence shows that mortality risk in haemodialysis patients undergoing VDR activation therapy is associated with increased level of serum calcium, phosphate and PTH (Teng *et al.* 2005). Although there is currently no scientific consensus on the detailed molecular understanding of selectivity of VDR activation, it is thought that it is attributed to selective interaction with different components of a transcriptional complex. In this notion, locally produced $1,25(OH)_2D$ in vascular cells could be compared to molecularly engineered selective VDR activator. However as good as it sounds in theory evidence shows that such pharmacological therapy mimics the endocrine system and therefore is not free of the aforementioned side effects. Even though a selective VDR activation by an active VDR activator - for

instance paricalcitol suppresses PTH and is manifested clinically by reduced hypercalcaemia and reduced hyperphosphotaemia (Sprague *et al.* 2003), the overall effect on progression of LVH and consequently morbidity in CKD is unchanged (Thadhani *et al.* 2012). Therefore, in principle the main benefit of auto/paracrine vitamin D system would be the reduced activity of locally produced 1,25(OH)$_2$D$_3$ on gastro-intestinal tract, kidney and bone. To date, the para/aurocrine vitamin D system in vasculature has not been studied in detail, nonetheless it has been postulated by some that the robustness of such system would be insufficient to support, mimic the endocrine system nor to maintain cellular health.

Synthesis of 1,25(OH)$_2$D in kidney fulfills both endocrine and autocrine actions. The thick ascending loop of Henle is an example of differentially controlled vitamin D system within a kidney. Firstly, the direct effects include control of calcium secretion by acting on CaSR, Na-Pi co-transporters, cell differentiation (Brown *et al.* 1996, Custer *et al.* 1994, Schwarz *et al.* 1998) and secondly vitamin D deficiency or low serum calcium do not result in decrease in *VDR* and *CYP24A1* mRNA, as opposed to for instance proximal convoluted tubule (Iida *et al.* 1995). In other tissues, such as the heart selective VDR activation in a rat CKD model was shown to decrease cardiomyocyte hypertrophy and cardiac dysfunction (Bodyak *et al.* 2007), where the pharmacological therapy also seemed to mimic the endocrine system. Although it is known that VDR activation in vasculature provides survival benefit, which is associated with a decrease in cardiovascular-related events (Shoji *et al.* 2004), existing data highlights further need to elucidate the mechanism of action of VDR signalling in vasculature. *Figure 4.1* depicts possible local hydroxylation of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ and subsequent effects of 1,25(OH)$_2$D$_3$ on downstream actions of VDR, 1α-OHase and 24-OHase. Previous evidence from *in vitro* studies showed that VDR activation in VSMCs plays an important role in cardiovascular

diseases. Gene chip microarray analysis provided a broad profile of the modulatory effects of $1,25(OH)_2D_3$ on human coronary artery SMCs. VDR activation by $1,25(OH)_2D_3$ was shown to downregulate mRNA and protein of atherotrombotic factors, such as plasminogen activator inhibitor-1 (an established marker of coronary heart disease) and thrombospondin-1 (Wu-Wong *et al.* 2007a).

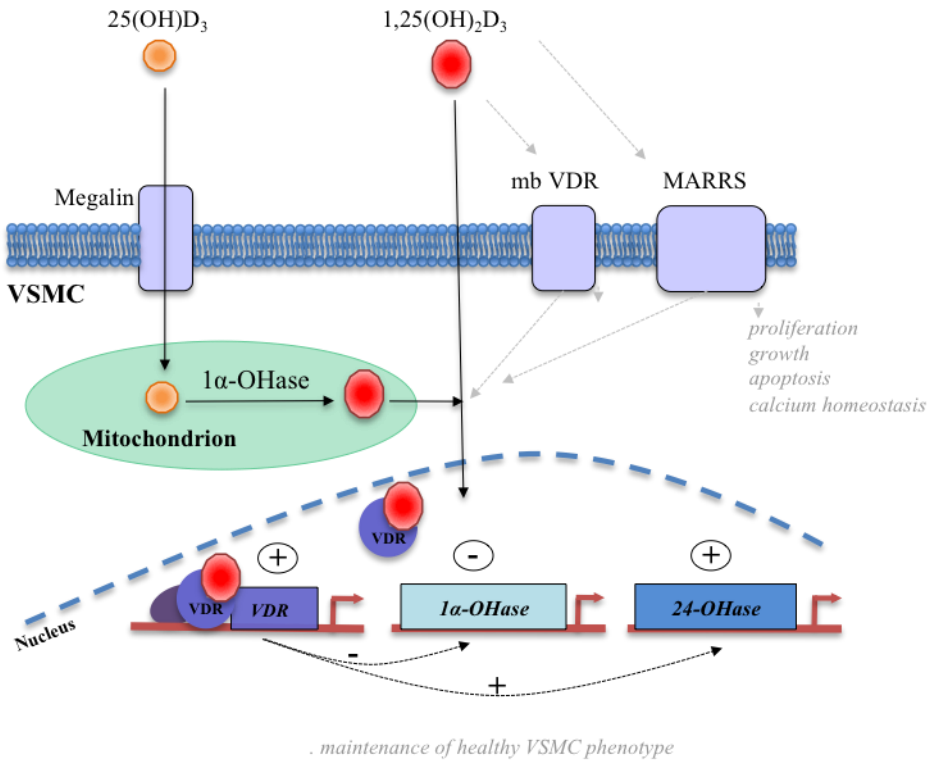


***Figure 4.1: Schematic Representation of Possible Local Hydroxylation of $25(OH)D_3$ to $1,25(OH)_2D_3$ and the subsequent effects of $1,25(OH)_2D_3$ on the downstream actions of the Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) in Vascular Smooth Muscle Cell (VSMC).*** *mb- membrane bound, MARRS- membrane associated rapid response steroid binding receptor, grey arrows – alternative mechanism of action, (-) inhibition, (+) upregulation.*

In the previous chapter I demonstrated that VDR, 1α-OHase and 24-OHase are all expressed in primary cultures of HAoSMCs and in human arteries, which confirmed that vasculature is a target tissue for $1,25(OH)_2D_3$, but also raised a question as to the role of the local vitamin D metabolism in maintaining vascular health, especially

taking into consideration my results which demonstrate that the expression of the vitamin D system is altered in the vessels of patients with CKD. Synthesis of $1,25(OH)_2D_3$ from $25(OH)D_3$ has been shown in human endothelial cells (Zehnder *et al.* 2002b), collecting duct cells of human nephron (Bland *et al.* 2001, Zehnder *et al.* 1999), prostate (Schwartz G. G. *et al.* 1998), parathyroid (Kawahara *et al.* 2008, Ritter *et al.* 2006) also in VSMCs (Somjen *et al.* 2005). The importance of $25(OH)D_3$ as a substrate for a local activation in VSMCs requires further investigation. It has been suggested, that subclinical vitamin D insufficiency (i.e. low 25(OH)D in the serum; 50-100 nM) is most likely to affect the extra-renal 1α-OHase activity, which is regulated differently to the renal enzyme (Hewison M. 2011).

In this chapter, the regulation of the vitamin D system by $1,25(OH)_2D_3$ in HAoSMCs, healthy and CKD human arteries was examined. Furthermore local 1α-OHase activity was analysed by quantifying local synthesis of $1,25(OH)_2D_3$ from $25(OH)D_3$ in HAoSMCs, healthy and CKD arteries (ongoing).

## 4.2 Results

### 4.2.1 Functional Vitamin D Signalling in HAoSMCs

***VDR is a Target for $1,25(OH)_2D_3$: Regulation of VDR, 1α-OHase and 24-OHase by $1,25(OH)_2D_3$ – Time Dependence***

HAoSMCs were treated with vehicle (0.1% ethanol) or $1,25(OH)_2D_3$ (10 nM) for 2, 4, 6 and 24 hours and mRNA expression was analysed by real-time RT-PCR. *VDR* mRNA was significantly upregulated 3.5-fold at 2 hours (*p=0.013*), 1.9-fold at 4 hours (*p=0.004*) and 1.8-fold at 6 hours (*p=0.04*) *(Figure 4.2A).*

*CYP27B1* mRNA expression was slightly, albeit significantly reduced at 6 hours *(p=0.045) (Figure 4.2B). CYP24A1* mRNA was increased by 30-fold at 2 hours *(p=0.01)* and 95-fold at 4 hours *(p=0.004)*; at 6 hours the induction in *CYP24A1* mRNA was 214-fold higher than in vehicle treated 6-hour control *(p=0.008) (Figure 4.2C).* At 24 hours *CYP24A1* mRNA level was almost back to the base level, possibly due to the complementation of the catabolic removal of $1,25(OH)_2D_3$.

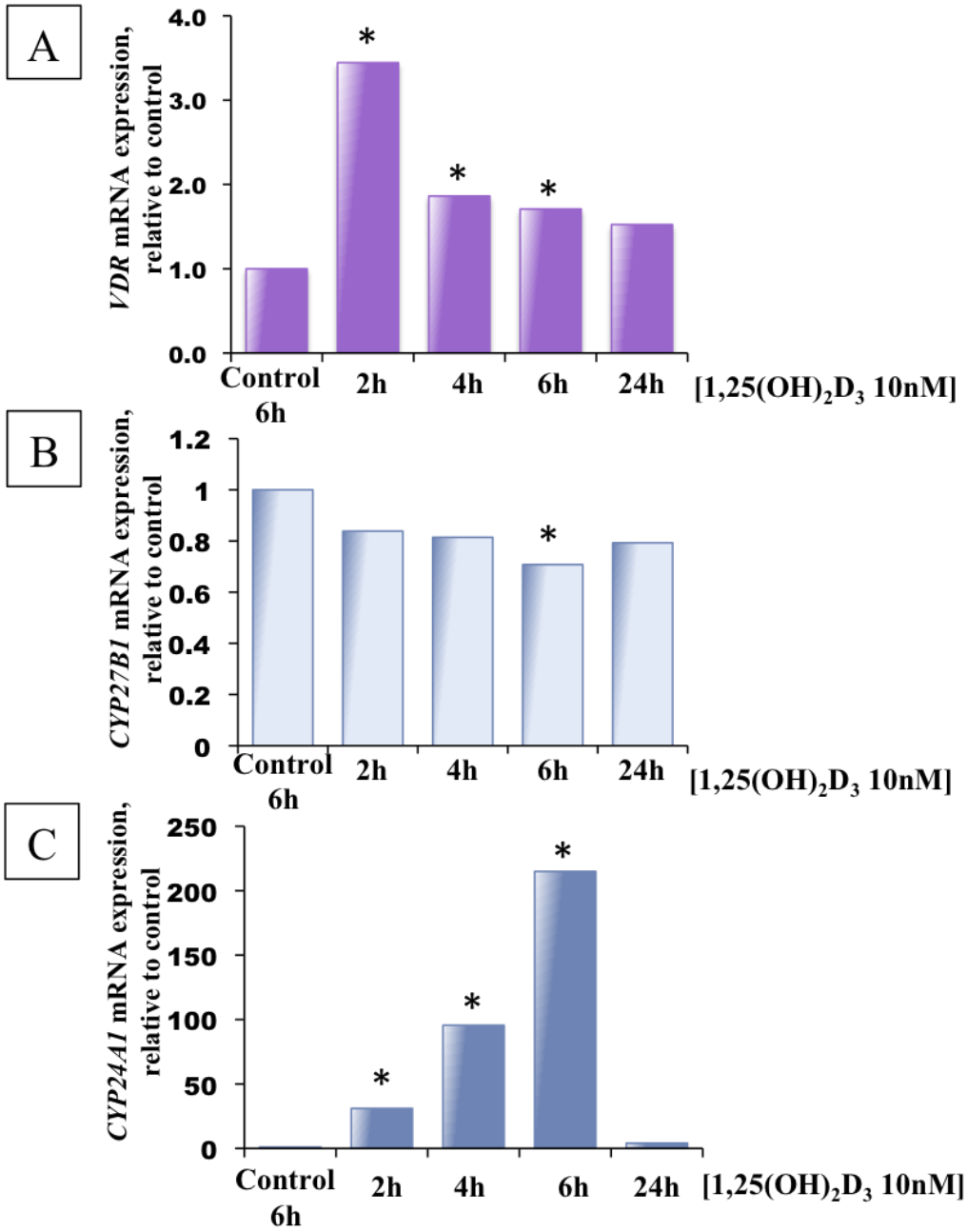


***Figure 4.2: The effect of 1,25(OH)$_2$D$_3$ on Vitamin D Receptor (VDR), 1α-Hydroxyalse (1α-OHase) and 24-Hydroxylase (24-OHase) mRNA expression in human aortic smooth muscle cells (HAoSMCs).*** *Cells were incubated with vehicle (0.1% ethanol) or 10nM 1,25(OH)$_2$D$_3$ for 2, 4, 6 and 24 hours. (A) VDR mRNA expression (B) 1α-OHase mRNA expression (C) 24-OHase mRNA expression, as demonstrated by real-time RT-PCR. VDR, 1α-OHase, 24-OHase mRNA levels were normalised to 18S rRNA. Results represent mean of relative mRNA levels, n=4-5; * $p < 0.05$; vs. 6h control, as determined by one-way ANOVA.*

To confirm that VDR, 1α-OHase and 24-OHase protein expression did not change over time when treated with vehicle, cells were cultured for 2, 4, 6, 24, 48 hours. *(Figure 4.3).* 6h treatment with vehicle (0.1% ethanol) was therefore used as control.

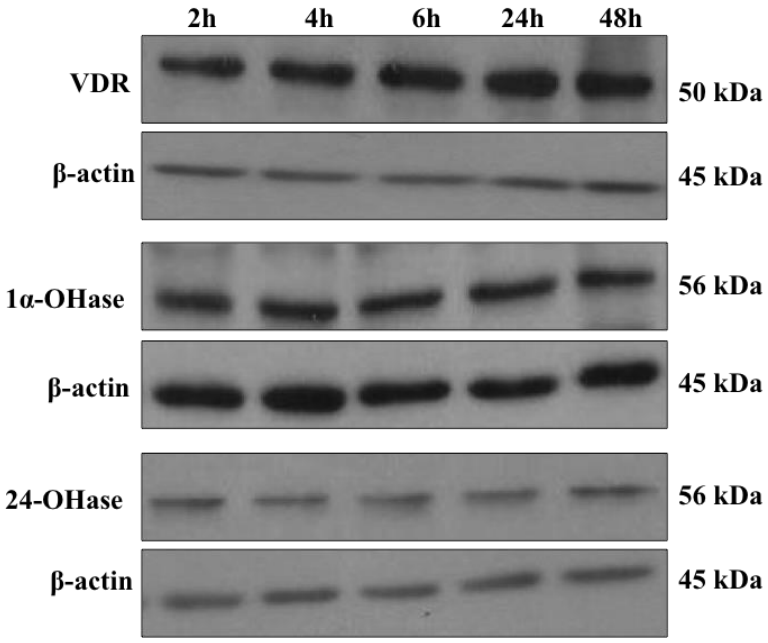


***Figure 4.3: Vitamin D Receptor (VDR), 1α-Hydroxyalse (1α-OHase) and 24-Hydroxyalse (24-OHase) protein expression in vehicle treated Human Aortic Smooth Muscle Cells (HAoSMC) over time.*** *Representative Western blots. Cells were incubated with vehicle (0.1% ethanol) for 2, 4, 6, 24 and 48 hours – no obvious changes in protein levels, over time, were observed.*

Western blot analyses of protein expression in HAoSMCs exposed to $1,25(OH)_2D_3$ (10 nM) for 6, 24 and 48 hours demonstrated that VDR protein was significantly elevated (1.8-fold) at 6 hours (*p=0.015*), compared to 6 hour vehicle treated control, confirming that VDR is a target for $1,25(OH)_2D_3$ *(Figure 4.4A).* 1α-OHase protein was significantly inhibited (0.2-fold) at 6 hours (*p=0.044*) after exposure to $1,25(OH)_2D_3$ (10 nM) *(Figure 4.4B).*

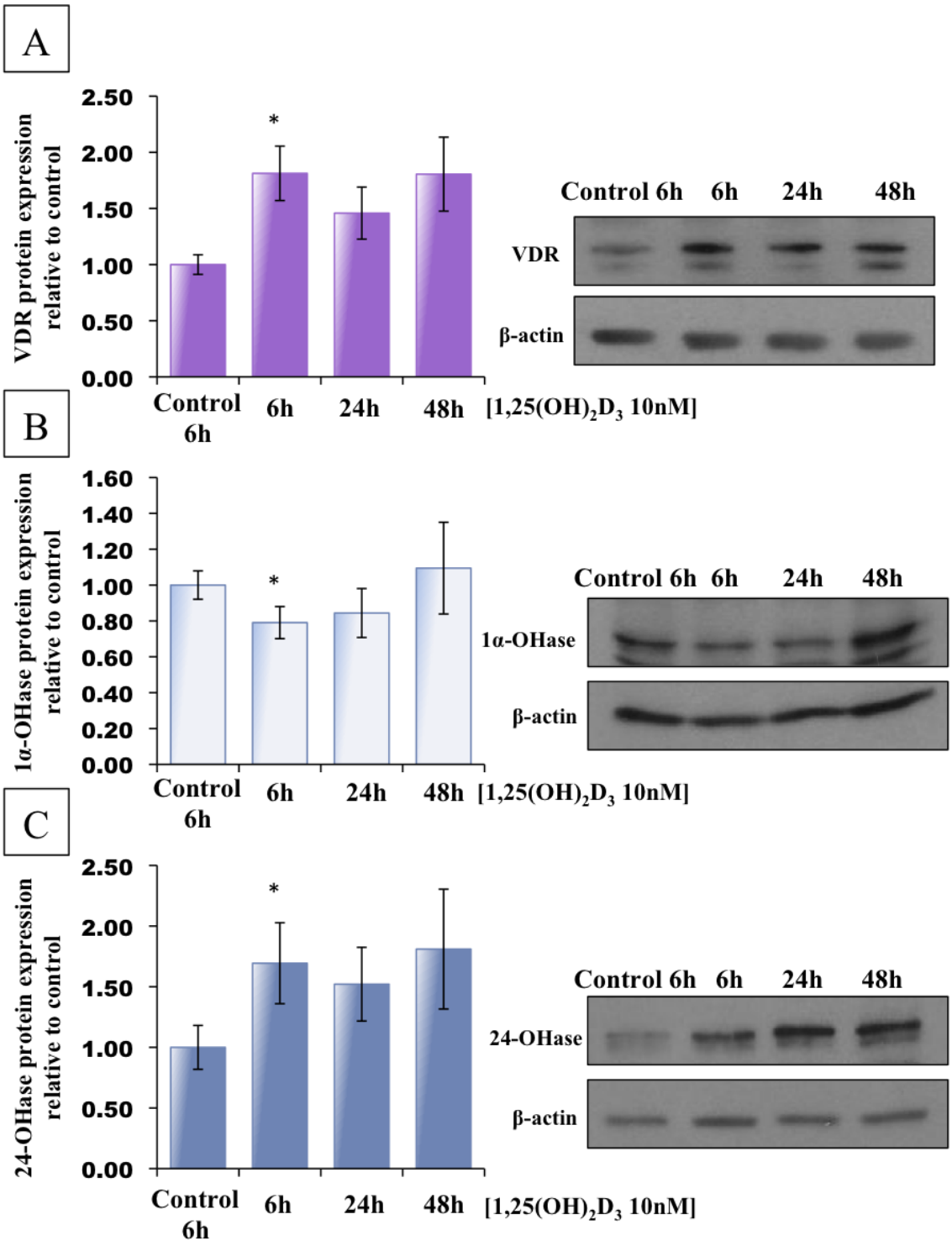


***Figure 4.4: The effect of 1,25(OH)$_2$D$_3$ on the Vitamin D receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxyalse (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *Cells were incubated with vehicle (0.1% ethanol) or 10 nM 1,25(OH)$_2$D$_3$ for 6, 24 and 48 hours. (A) VDR protein expression (B) 1α-OHase protein expression (C) 24-OHase protein expression, as demonstrated by Western blot analyses. Results represent mean of relative protein levels ± SEM, n=4-8; * p < 0.05; vs. control, as determined by one-way ANOVA. Results were normalised to β-actin levels. Next to each graph are the representative Western blots.*

24-OHase protein was significantly upregulated at 6 hours *(p=0.01)*, just like 1α-OHase and VDR *(Figure 4.4C).*

In summary, 1,25(OH)$_2$D$_3$ (10 nM) activated *VDR* gene transcription by 2 hours and VDR protein synthesis by 6 hours, which consequently modulated the expression of the downstream genes – *CYP27B1* (by 6 hours) and *CYP24A1* (by 2 hours, 6 hours and 24 hours). *VDR* and *CYP27B1* mRNA induction appeared to be tightly controlled by increase in *CYP24A1* mRNA, via a negative feedback. Changes in VDR, 1α-OHase and 24-OHase protein expression by 6 hours reflected changes in mRNA. By 24 hours, no significant changes in protein expression were recorded, although the protein expression appeared to follow the trend described for mRNA (initial increase in VDR early after 1,25(OH)$_2$D$_3$ stimulation, increase in 24-OHase and drop in 1α-OHase, further increase in 24-OHase, VDR and 1α-OHase back to control levels).

### *The VDR is a Target for 1,25(OH)$_2$D$_3$: Regulation of the VDR, 1α-OHase and 24-OHase by 1,25(OH)$_2$D$_3$ – Dose Dependence*

In order to obtain a broader picture of the dynamics of VDR activation, the effect of increasing concentrations of 1,25(OH)$_2$D$_3$ (0.1, 1, 10, 100 nM; 6 hours was chosen based on my earlier results where 10 nM 1,25(OH)$_2$D$_3$ significantly altered expression of VDR, 1α-OHase and 24-OHase protein at 6 hours, see *Figure 4.2*) on expression of *VDR, CYP27B1* and *CYP24A1* mRNA and protein was examined in HAoSMCs.

Real-time RT-PCR analyses confirmed that *VDR* mRNA was significantly increased (10-fold) by 1,25(OH)$_2$D$_3$ (10 nM) *(p=0.040) (Figure 4.5A), CYP27B1* mRNA was

significantly decreased, by 0.5-fold *(p=0.038)* by 1,25(OH)$_2$D$_3$ (10 nM) (also by 1 or 100 nM 1,25(OH)$_2$D$_3$; *p=0.021*, *p=0.042* respectively) *(Figure 4.5B),* conversely *CYP24A1* mRNA was elevated by 1000-fold *(p=0.021)* by 1,25(OH)$_2$D$_3$ (10 nM) (also by 100 nM 1,25(OH)$_2$D$_3$*;* 4000-fold*; p=0.033*) *(Figure 4.5C).* When *CYP27B1* mRNA expression was reduced by over a quarter, *CYP24A1* mRNA was increased by over 4000-fold.

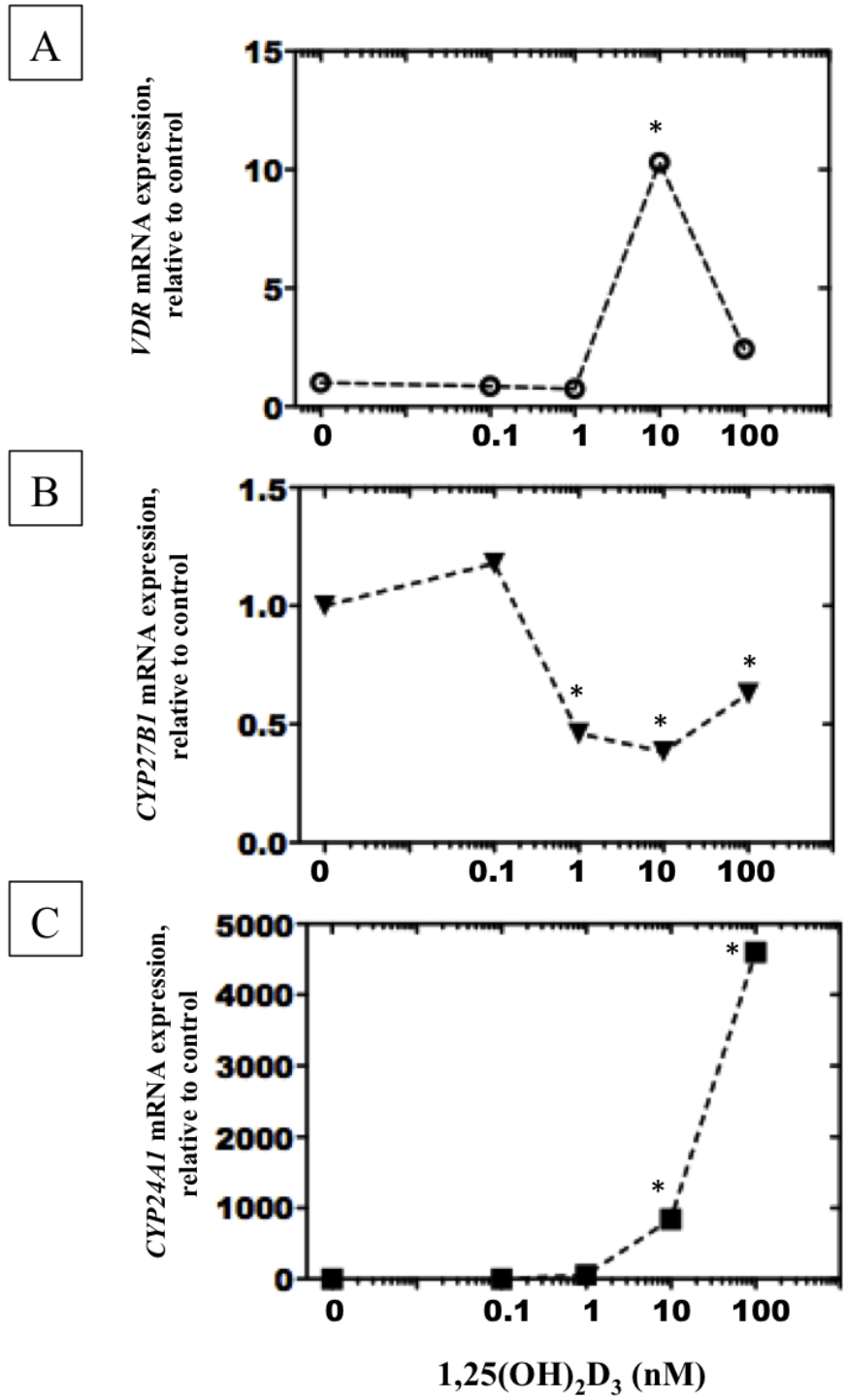


***Figure 4.5: The effect of 1,25(OH)$_2$D$_3$ on 1α-Hydroxylase (CYP27B1), 24-Hydroxylase (CYP24A1) and Vitamin D Receptor (VDR) mRNA expression in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *Cells were incubated with vehicle (0.1% ethanol, control), or 0.1, 1, 10 or 100 nM 1,25(OH)$_2$D$_3$ for 6 hours. (A) CYP27B1 mRNA expression (B) CYP24A1 mRNA expression (C) VDR mRNA expression, as demonstrated by real-time RT-PCR. All readings were normalised to 18S rRNA levels. Results represent mean of relative mRNA levels and were plotted against treatment concentrations, n=3-4; * p < 0.05; vs. control, as determined by one-way ANOVA.*

In general, changes induced by 1,25(OH)$_2$D$_3$ in mRNA expression corresponded to the changes detected in protein expression for VDR, 1α-OHase and 24-OHase, with a few exceptions. There was no significant decrease in 1α-OHase (nor the significant increase in 24-OHase) protein expression at higher concentration of 1,25(OH)$_2$D$_3$ (100 nM) *(Figure 4.7).* Otherwise, at 6 hours VDR protein expression was significantly induced (over 2-fold) by 10 nM or 100 nM 1,25(OH)$_2$D$_3$ *(p=0.021, p=0.027 respectively),* compared to control *(Figure 4.6).* 1α-OHase protein level dropped significantly as a result of treatment with 0.1 nM, 1 nM or 10 nM of 1,25(OH)$_2$D$_3$ *(p=0.030, p=0.021, p=0.038 respectively),* as compared to control *(Figure 4.7A).* 24-OHase protein was significantly induced 1.36-fold by 10 nM 1,25(OH)$_2$D$_3$, compared to control *(p=0.017) (Figure 4.7B).*

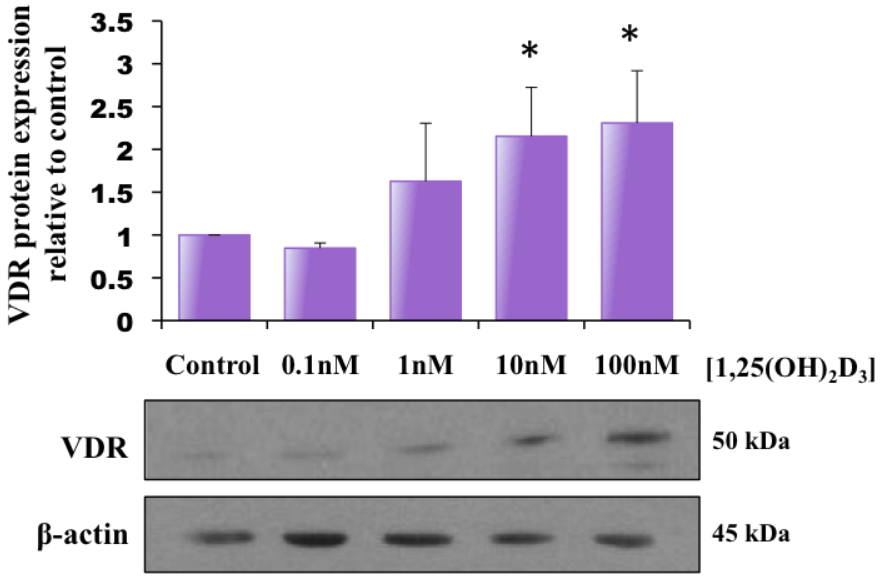


***Figure 4.6: The effect of 1,25(OH)$_2$D$_3$ on the Vitamin D Receptor (VDR) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *Cells were incubated with vehicle (0.1% ethanol, control) or 0.1, 1, 10 or 100 nM 1,25(OH)$_2$D$_3$ for 6 hours. VDR protein expression levels were normalised to β-actin. Results represent mean of relative protein levels ±SEM, n= 3-9; * p < 0.05; vs. control, as determined by one-way ANOVA. Below the graph are the representative Western blots.*

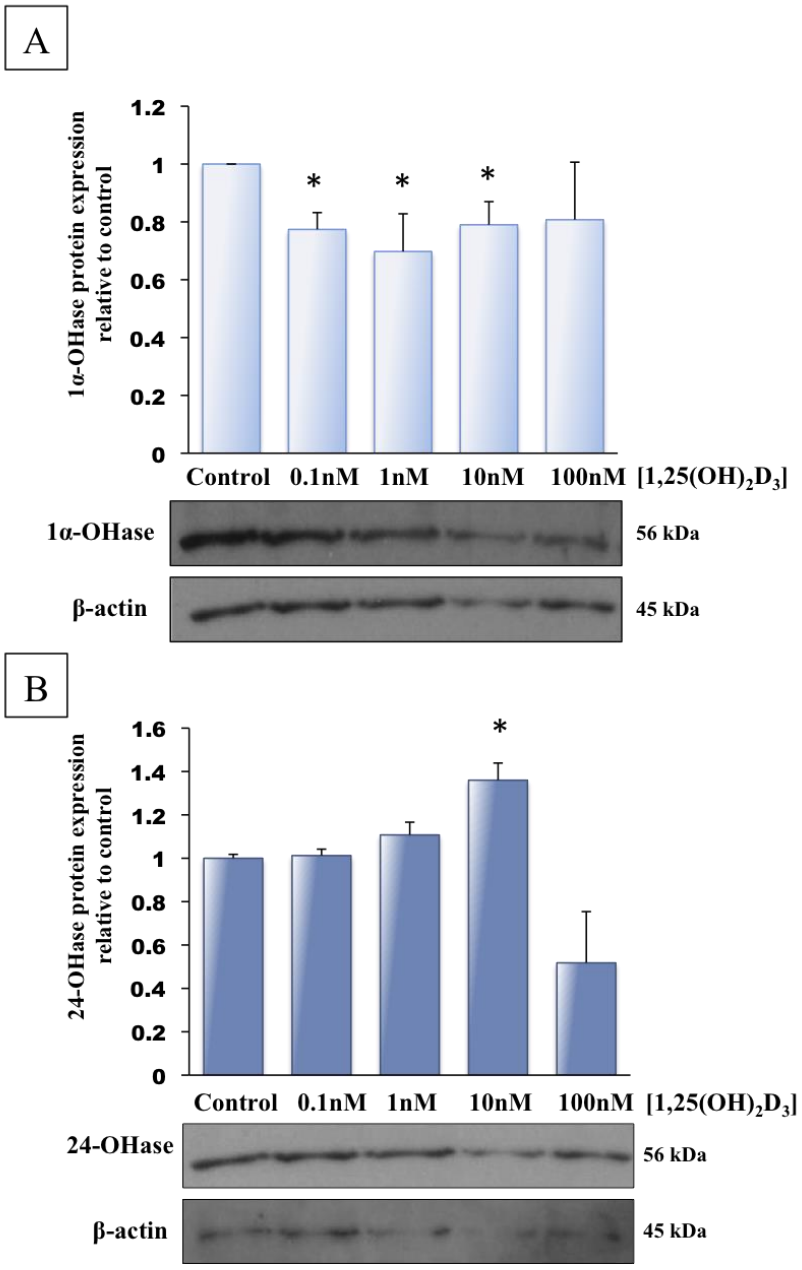


***Figure 4.7: The effect of increasing concentrations of 1,25(OH)$_2$D$_3$ on 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *Cells were incubated with vehicle (0.1% ethanol, control) or 0.1, 1, 10 or 100 nM 1,25(OH)$_2$D$_3$ for 6 hours. (A) 1α-OHase protein expression (B) 24-OHase protein expression. 1α-OHase and 24-OHase protein levels were normalised to β-actin. Results represent mean of relative protein levels ± SEM, n= 3-9, * p < 0.05; vs. control, as determined by one-way ANOVA. Below the graphs are the representative Western blots.*

### 4.2.2 Local Synthesis of $1,25(OH)_2D_3$ from $25(OH)D_3$ in HAoSMCs – *Time Dependence*

In order for the treatment with $25(OH)D_3$ to act via the VDR, $25(OH)D_3$ has to first be converted to $1,25(OH)_2D_3$. HAoSMCs were treated with $25(OH)D_3$ (100 nM) for 2, 4, 6 and 24 hours and changes in *VDR*, *CYP27B1* and *CYP24A1* mRNA were examined by real-time RT-PCR. Results indicated that *VDR* mRNA expression was significantly induced (3.7-fold) at 2 hours *(p=0.007)* and by (2.6-fold) at 4 hours *(p=0.047)* compared to control. Interestingly, the mRNA levels dropped to control levels by 6 hours and significantly dropped below control levels (0.6-fold) by 24 hours *(p=0.007) (Figure 4.8A). CYP27B1* mRNA expression was significantly inhibited at 6 hours *(p=0.042) (Figure 4.8B).* $25(OH)D_3$ (100 nM) significantly induced *CYP24A1* mRNA at 2 hours (18.9-fold) *(p=0.021),* at 4 hours (143.3-fold) *(p=0.049),* at 6 hours (923.3-fold) *(p=0.009)* and at 24 hours (153.8-fold) *(p=0.046) (Figure 4.8C).* These results demonstrate local synthesis of $1,25(OH)_2D_3$ from $25(OH)D_3$.

Western blot analyses demonstrated that $25(OH)D_3$ (100 nM) did not induce VDR protein at 48 hours *(p=0.28) (Figure 4.9A).* 1α-OHase protein was significantly induced 1.7-fold at 24 hours, compared to control *(p=0.049) (Figure 4.9B).*

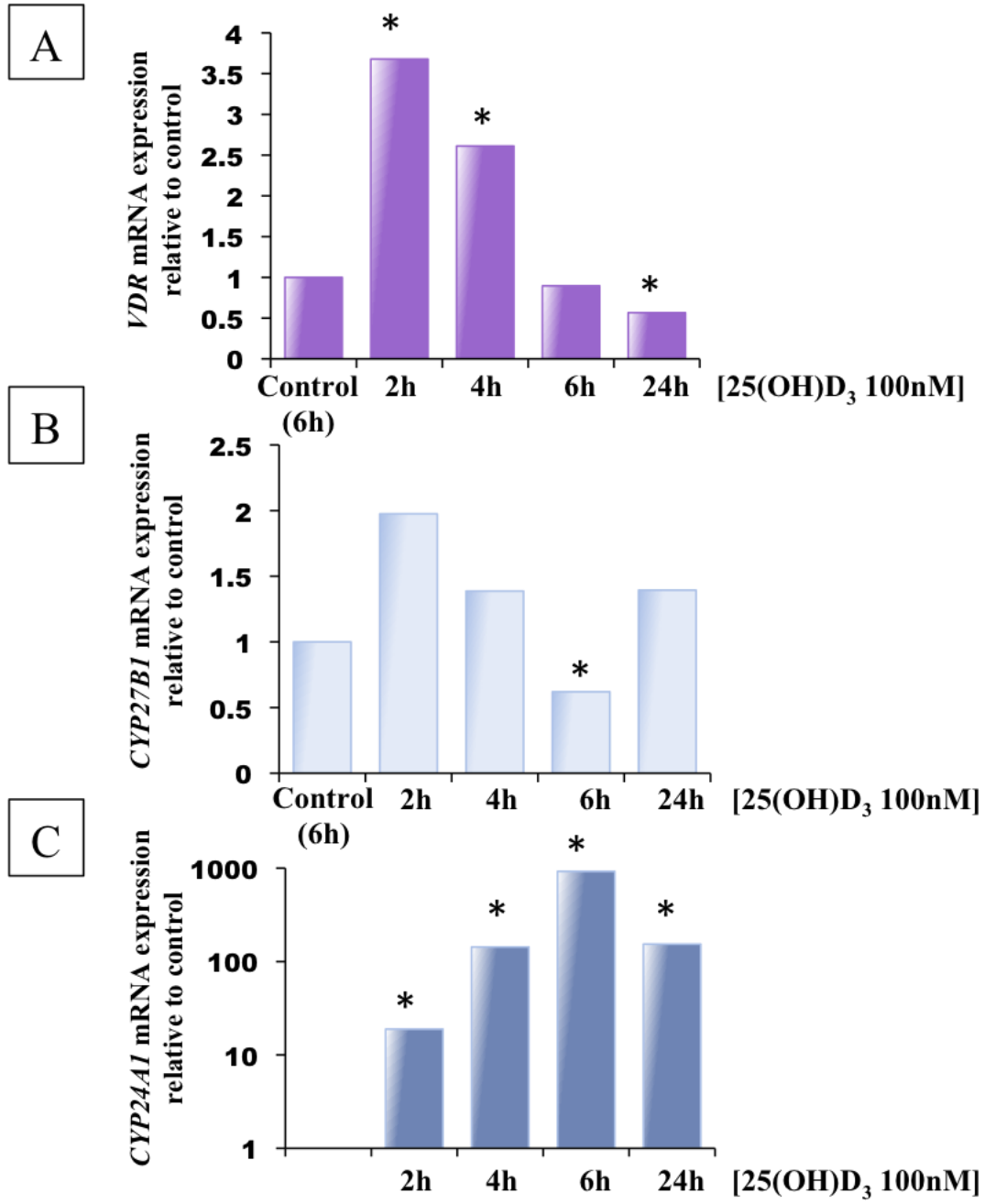


***Figure 4.8: The effect of 25(OH)D$_3$ on Vitamin D Receptor (VDR), 1α-Hydroxylase (CYP27B1) and 24-Hydroxylase (CYP24A1) mRNA expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *Cells were incubated with vehicle (0.1% ethanol, control) or 100 nM 25(OH)D$_3$ for 2, 4, 6, 24 and 48 hours. (A) VDR mRNA expression (B) CYP27B1 mRNA expression (C) CYP24A1 expression, as demonstrated by real-time RT-PCR. VDR, CYP27B1 and CYP24A1 mRNA levels were normalised to 18S rRNA levels. Results represent mean of relative mRNA levels; n =4-5, * p < 0.05; vs. control, as determined by one-way ANOVA.*

The increase in 1α-OHase protein was preceded by 1.4-fold raise (not-statistically significant) in *CYP27B1* mRNA at 24 hours (the peak could have occurred at an earlier time point – for instance 12 hours, which would allow a sufficient lag for the subsequent protein synthesis by 24 hours) *(Figure 4.8B).* An increase in 1α-OHase protein at 24 hours could indicate that there was a need for 1α-OHase protein to outcompete 24-OHase due to an abundance of substrate: 25(OH)D$_3$. 24-OHase protein expression was not significantly different *(Figure 4.9C).*

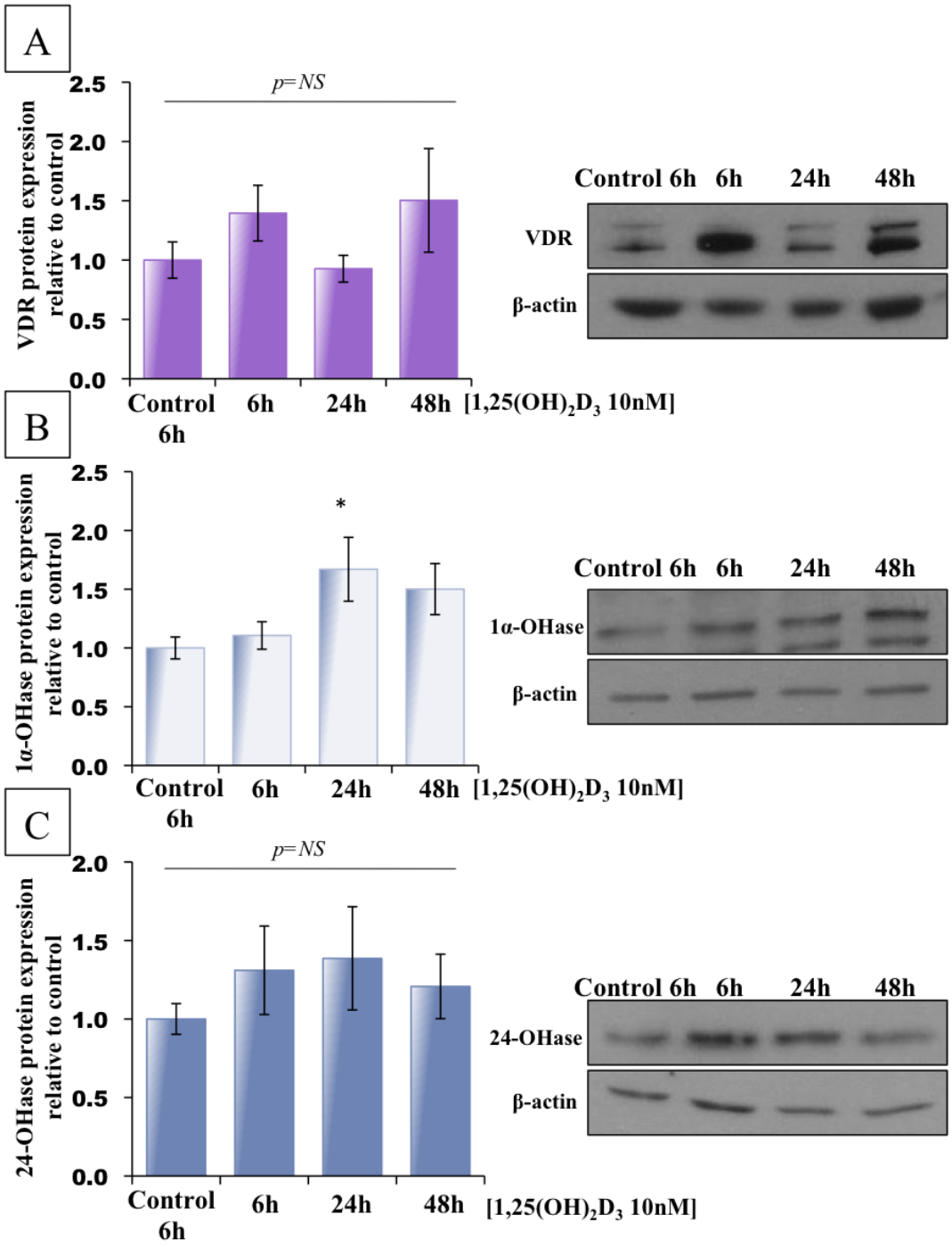


***Figure 4.9: The effect of 25(OH)D$_3$ on the Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxyalse (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *Cells were incubated with vehicle (0.1% ethanol) or 10 nM 1,25(OH)$_2$D$_3$ for 6, 24 and 48 hours. (A) VDR protein expression (B) 1α-OHase protein (C) 24-OHase protein expression, as demonstrated by Western blot analyses. VDR, 1α-OHase, 24-OHase protein levels were normalised to β-actin. Results represent mean of relative protein levels ± SEM, n=4-8; * p < 0.05; vs. 6h control, as determined by one-way ANOVA; NS – not significant. Next to each graph are the representative Western blots.*

### 4.2.3 Local Synthesis of $1,25(OH)_2D_3$ from $25(OH)D_3$ in HAoSMCs – *Dose Dependence*

In order to examine the impact of substrate availability on VDR signalling dynamics, HAoSMCs were incubated with vehicle (0.1% ethanol) or increasing concentrations of $25(OH)D_3$ (0.1, 1, 10, 100 nM for 6 hours – based on my earlier results). *VDR*, *CYP27B1* and *CYP24A1* mRNA and protein levels were analysed.

Real-time RT-PCR analyses showed that *CYP27B1* mRNA was significantly decreased 0.5-fold by 100 nM $25(OH)D_3$ *(p=0.03)* and 0.6-fold by 1000 nM *(p=0.03) (Figure 4.10B). CYP24A1* mRNA expression was significantly elevated 2000-fold by 100 nM $25(OH)D_3$ (p=0.018) and 1000-fold by 1000 nM $25(OH)D_3$ *(p=0.014) (Figure 4.10C). VDR* mRNA levels were not significantly different (*Figure 4.10A).*

Western blot analyses revealed, that at 6 hours VDR protein expression was not significantly changed *(Figure 4.10A).* As seen previously, 100 nM $25(OH)D_3$ did not induce a significant change in 1α-OHase protein expression. Also, no changes were induced by higher or lower concentrations of $25(OH)D_3$ (1, 10, 1000 nM) *(Figure 4.11B).* There was a lot of variation in 24-OHase protein expression between individual experiments, which was reflected in large SEM values.

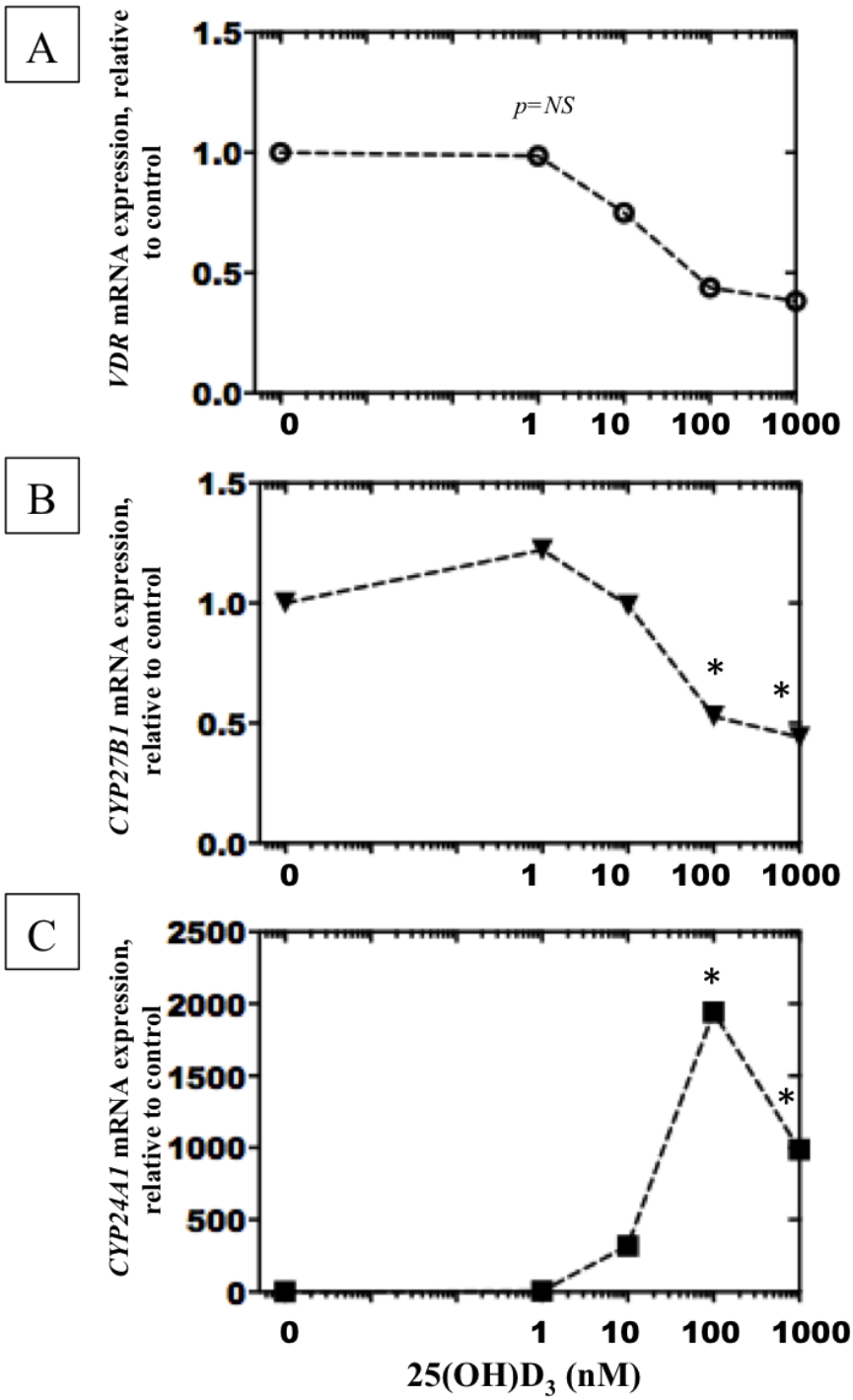


***Figure 4.10: The effect of increasing concentrations of 25(OH)D$_3$ on the Vitamin D Receptor (VDR), 1α-Hydroxylase (CYP27B1) and 24-Hydroxylase (CYP24A1) mRNA expression in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *Cells were incubated with vehicle (0.1% ethanol, control) or 1, 10, 100 or 1000 nM 25(OH)D$_3$ for 6 hours. (A) VDR mRNA expression (B) CYP27B1 mRNA expression (C) CYP24A1 mRNA expression, as demonstrated by real-time RT-PCR. Values were normalised to 18S rRNA levels. Results represent mean of relative mRNA levels, n =4-5;* p < 0.05; vs. control, as determined by one-way ANOVA; NS –not significant.*

1000 nM 25(OH)$D_3$ had no effect on 24-OHase protein expression *(Figure 4.11C).*

It is possible that changes in protein do not reflect activity.

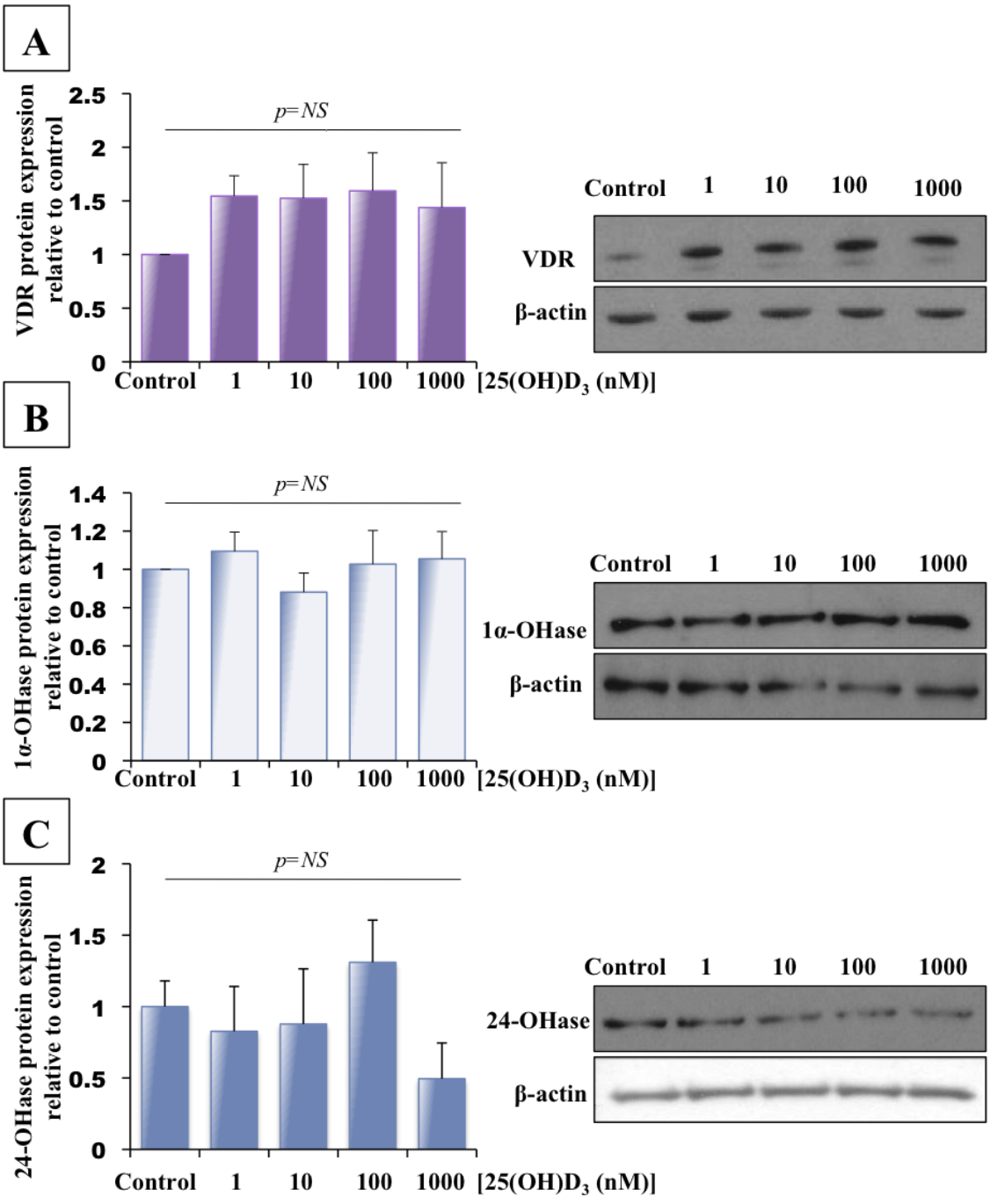


***Figure 4.11: The effect of increasing concentrations of 25(OH)$D_3$ on Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *Cells were incubated with vehicle (0.1% ethanol, control) or 1, 10, 100 or 1000 nM 25(OH)$D_3$ for 6 hours. (A) VDR, (B) 1α-OHase, (C) 24-OHase protein, as demonstrated by Western blot analyses. VDR, 1α-OHase and 24-OHase protein levels were normalised to β-actin. Results represent mean of relative protein levels ± SEM, n= 3-*

*9, * p < 0.05; vs. control, as determined by one-way ANOVA. Next to each graph are the representative Western blots.*

### *Differences in 24-OHase-Driven Catabolism might be Substrate Dependent*

High local concentration of $1,25(OH)_2D_3$ (10nM) or very high (100 nM) (equivalent to 10 X physiological levels in circulation ~ 0.11 nM) significantly induced *CYP24A1* mRNA at 6 hours, as discussed earlier. Similarly, high concentration of $25(OH)D_3$ (100 nM) or very high (1000 nM) (physiological level in circulation ~ 50 nM) also significantly induced *CYP24A1* mRNA expression by 6 hours. However, when *CYP24A1* mRNA increase was almost logarithmic in response to increasing doses of $1,25(OH)_2D_3$ *(Figure 4.12A),* it appeared that highest dose of $25(OH)D_3$ (1000 nM) resulted in the 1000-fold drop, compared to *CYP24A1* mRNA levels induced by 100 nM $25(OH)D_3$ *(Figure 4.12B).*

24-OHase protein expression by 6 hours only partially reflected changes in mRNA. $1,25(OH)_2D_3$ (10 nM and 100 nM) induced no significant change in 24-OHase *(Figure 4.13).*

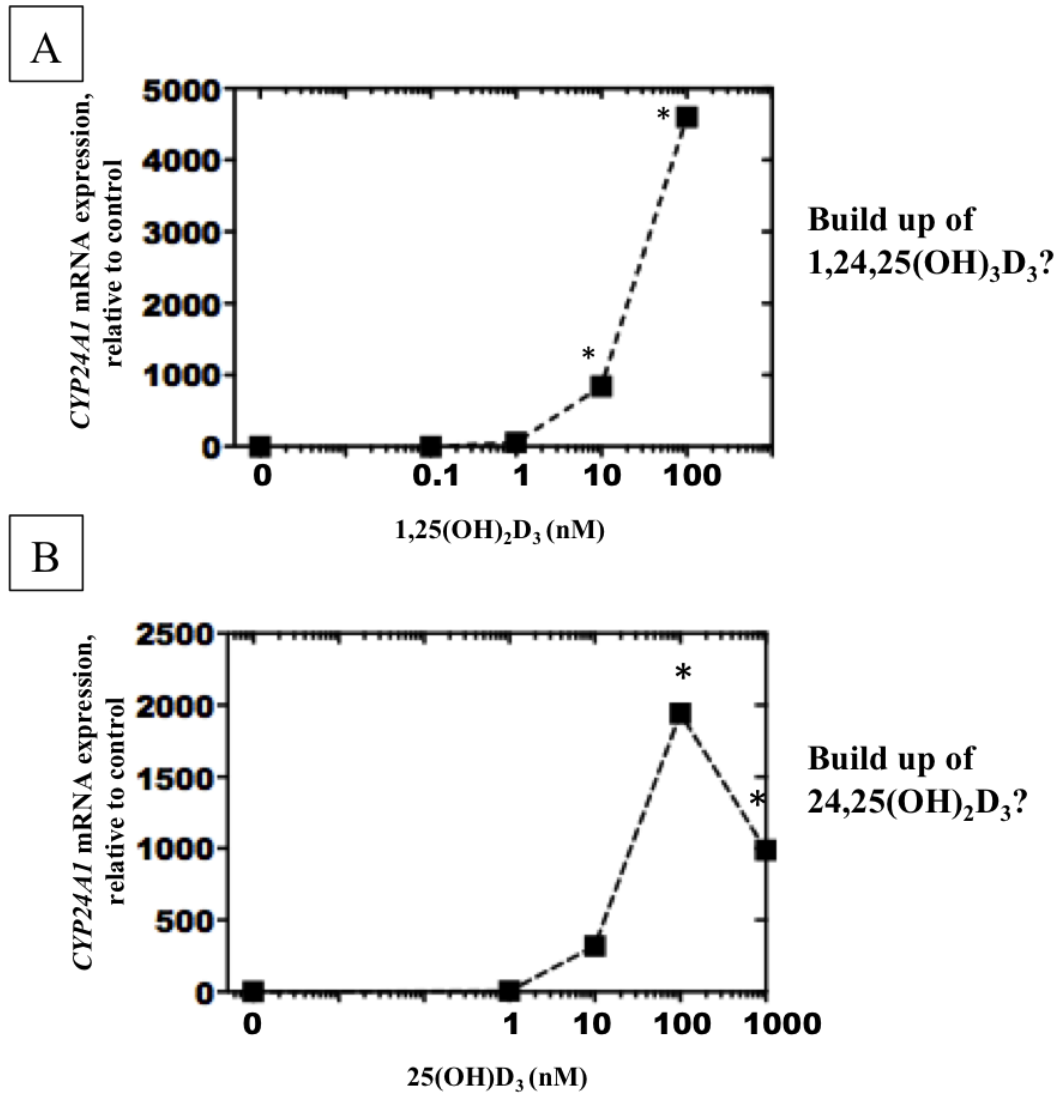


***Figure 4.12: The effect of increasing concentrations of $1,25(OH)_2D_3$ or $25(OH)D_3$ on 24-Hydroxylase (CYP24A1) mRNA expression in Human Aortic Smooth Muscle Cells (HAoSMCs)***. *Cells were incubated with vehicle (0.1% ethanol, control) or 0.1, 1, 10, 100 nM $1,25(OH)_2D_3$ or 1, 10, 100 or 1000 nM $25(OH)D_3$ for 6 hours. (A) CYP24A1 mRNA expression was significantly increased by 10 and 100 nM $1,25(OH)_2D_3$, whereas (B) CYP24A1 mRNA was significantly induced by 100 nM and 1000 nM $25(OH)D_3$. Readings were normalised for 18S rRNA levels. Results represent mean of relative mRNA levels, n =4-5; * $p < 0.05$; vs. untreated control, as determined by one-way ANOVA.*

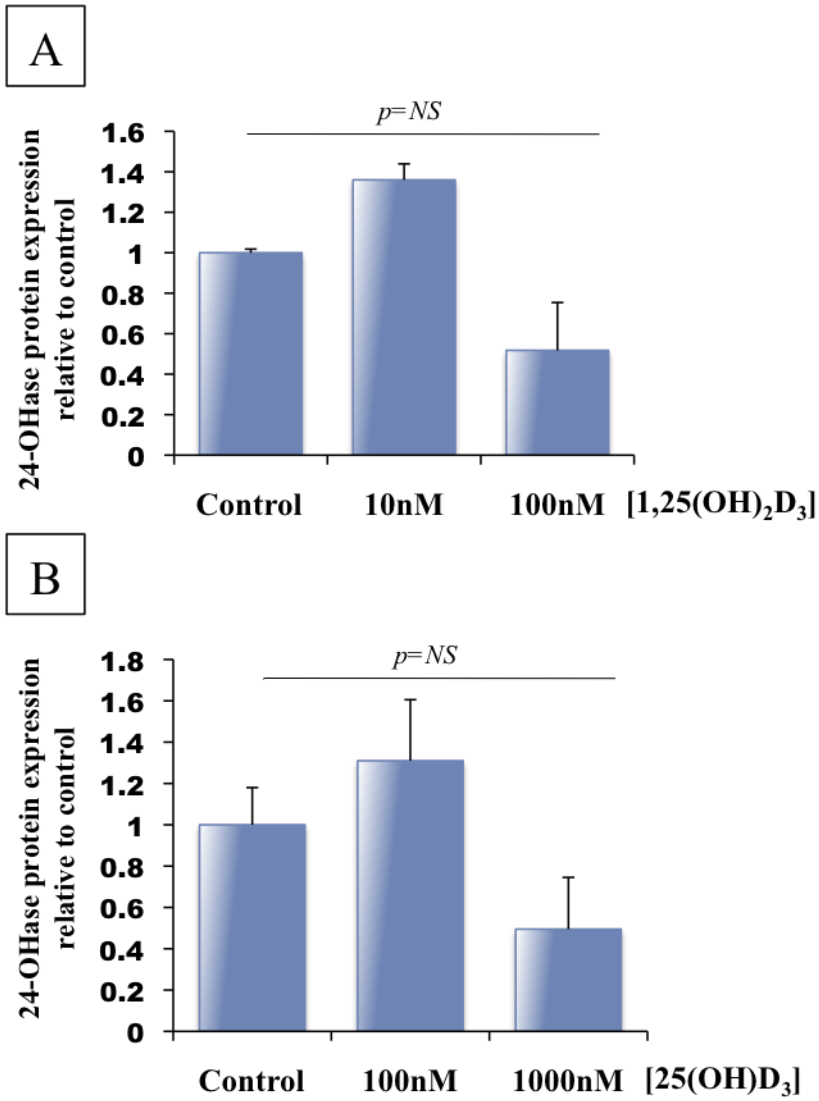


***Figure 4.13: The effect of increasing concentrations of 1,25(OH)$_2$D$_3$ or 25(OH)D$_3$ on 24-Hydroxylase (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *(A) Cells were incubated with vehicle (0.1% ethanol, control) or 10 or 100 nM 1,25(OH)$_2$D$_3$ for 6 hours (B) Cells were incubated with vehicle (0.1% ethanol, control) or 100 or 1000 nM 25(OH)D$_3$ for 6 hours. Protein expression as demonstrated by Western blot analyses. 24-OHase protein levels were normalised to β-actin. Results represent mean of relative protein levels, n =4-5; * p < 0.05; vs. control, as determined by one-way ANOVA.*

### 4.2.4 Vitamin D Signalling in Human Arteries

#### *The Effect of 1,25(OH)$_2$D$_3$ on VDR, CYP27B1 and CYP24A1 mRNA Expression in Healthy and CKD Human Artery*

To translate my previous *in vitro* findings to organ, artery culture and to confirm that the normal functioning of vitamin D system in healthy human artery is altered in

CKD, real-time RT-PCR analyses were carried out using RNA isolated from arterial explants of healthy and CKD patients. Arterial explants from both groups (n = 11 healthy, n = 16 CKD) were washed in growth medium and incubated with 10 nM 1,25(OH)$_2$D$_3$ for 48 hours allowing time for the steroid to penetrate the artery (*Figure 2.3*). All real time RT-PCR analyses human artery data were provided by Dr Guerman Molostvov.

In healthy arteries, the basal expression of *VDR* mRNA was significantly higher (50%) than in arteries from patients undergoing kidney transplantation *(p<0.01) (Figure 4.15A). CYP27B1* mRNA in CKD vessels was increased by almost 100%, compared to levels in healthy arteries *(p<0.01)* (*Figure 4.14B*). No significant differences in expression of *CYP24A1* mRNA between healthy and CKD arteries were detected *(Figure 4.14C).*

Real-time analyses indicated a very large increase (470%) in *VDR* mRNA, as a response to 1,25(OH)$_2$D$_3$ in healthy arteries compared to healthy untreated control *(p<0.05)* (*Figure 4.14A*). This induction was not as strong in CKD, with only a 50% increase in *VDR* mRNA expression *(p<0.01) (Figure 4.14A).* Treatment with 1,25(OH)$_2$D$_3$ (10 nM) lead to 24% decrease in *CYP27B1* mRNA in healthy arteries, compared to healthy untreated arteries, however the change was not statistically significant *(Figure 4.14B).* Conversely, in CKD arteries, 1,25(OH)$_2$D$_3$ (10 nM) induced a 22% increase in *CYP27B1* mRNA expression, compared to untreated CKD arteries, however the increase was not statistically significant. *CYP27B1* mRNA basal levels in healthy untreated arteries were 94% lower than in CKD *(p<0.01). CYP24A1* mRNA levels were 24% lower in CKD arteries, compared to levels in healthy untreated arteries. At 48 hours, 1,25(OH)$_2$D$_3$ induced a 2096% increase in *CYP24A1* mRNA in healthy arteries, compared to healthy untreated arteries

*(p<0.01).* In CKD arteries 1,25(OH)$_2$D$_3$ (10 nM) induced a stunning increase (34352%) in *CYP24A1* mRNA levels *(p<0.0001). CYP24A1* mRNA induction, as a result of 1,25(OH)$_2$D$_3$ (10 nM) 48h treatment, was 32242% higher in CKD than in healthy arteries *(p<0.001) (Figure 4.14C).*

### *The Relation between CYP27B1 and CYP24A1 mRNA and Protein Expression in Normal Human and CKD Arteries*

In chapter 3 I showed that VDR protein expression was not significantly different in CKD arteries, compared to healthy arteries *(Figure 3.5).* Whereas mRNA data showed a significant decrease in *VDR* expression in CKD (50%) compared to healthy arteries *(p<0.01) (Figure 4.14A).*

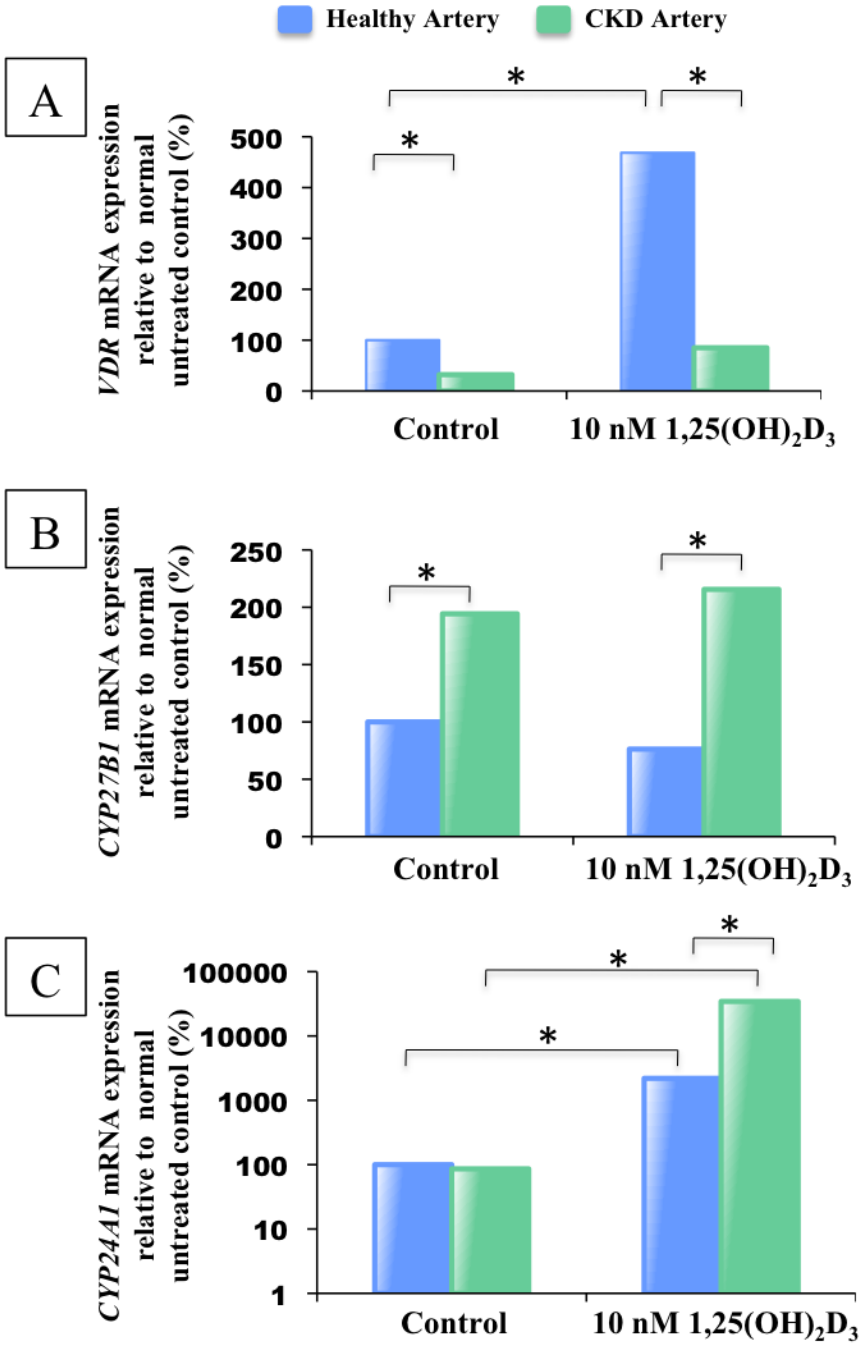


***Figure 4.14: The effect of 1,25(OH)$_2$D$_3$ on the Vitamin D Receptor (VDR), 1α-Hydroxylase (CYP27B1) and 24-Hydroxyalse (CYP24A1) mRNA expression in Healthy and Chronic Kidney Disease (CKD) arteries.*** *Arteries were incubated with 10 nM 1,25(OH)$_2$D$_3$ for 48 hours. Differences in mRNA expression were examined by real-time RT-PCR analysis (normalised to 18S rRNA levels). Note different scales. (A) VDR mRNA expression, (B) CYP27B1 mRNA expression (C) CYP24A1 mRNA expression. Results represent mean of relative mRNA levels; n =11 healthy, 16 CKD, * $p < 0.05$; vs. healthy untreated control and vs. healthy treated control, as determined by one-way ANOVA.*

Although, *CYP27B1* mRNA expression was significantly increased in CKD, compared to healthy arteries (98%) *(p<0.01) (Figure 4.14A),* this was not reflected in protein levels, which were significantly lower in CKD, compared to control arteries *(p=0.048)* (Figure 3.6). *CYP24A1* mRNA levels were 24% lower in CKD than in control arteries, however the difference was not statistically significant *(Figure 4.14C).* This was not reflected in 24-OHase protein expression, as earlier results showed that 24-OHase protein expression was increased by over a quarter in CKD, compared to healthy arteries, here too, the change was not statistically significant *(p=0.12) (Figure 3.7).* Despite some of the changes being statistically insignificant, the uncoupling between *CYP27B1* and *CYP24A1* mRNA and protein levels in healthy and CKD arteries may be a true phenomenon and a larger sample size is required to confirm this finding.

### 4.2.5 The Assessment of 1α-OHase Activity in HAoSMCs and in Healthy and CKD Artery

HAoSMCs and human artery samples (healthy, n=8 and CKD, n=11) were collected and treated as described in chapter 2.2.4.1 to determine the synthesis of $1,25(OH)_2D_3$.

Synthesis of $1,25(OH)_2D_3$ was determined by treating samples for 5 hours with 100 nM $25(OH)D_3$. Secreted $1,25(OH)_2D_3$ was assayed by EIA (Dr Guerman Molostvov). All samples synthesised $1,25(OH)_2D_3$ indicating 1α-OHase activity.

HAoSMCs are able to synthesise $1,25(OH)_2D_3$ (118.9 fmoles of $1,25(OH)_2D_3$/mg protein (/5 hours) indicating 1α-OHase activity *(Figure 4.15A).* Data from the arterial samples indicated that although $1,25(OH)_2D_3$ synthesis could be detected levels were significantly lower than that seen in HAoSMCs (*Figure 4.15*), note different scales.

In addition, 1,25(OH)$_2$D$_3$ production was significantly reduced in arteries from CKD patients (3.5 fmoles of 1,25(OH)$_2$D$_3$/mg protein/5h vs. 17.6 fmoles of 1,25(OH)$_2$D$_3$/mg protein/5h), *p=0.036 (Figure 4.15B).*

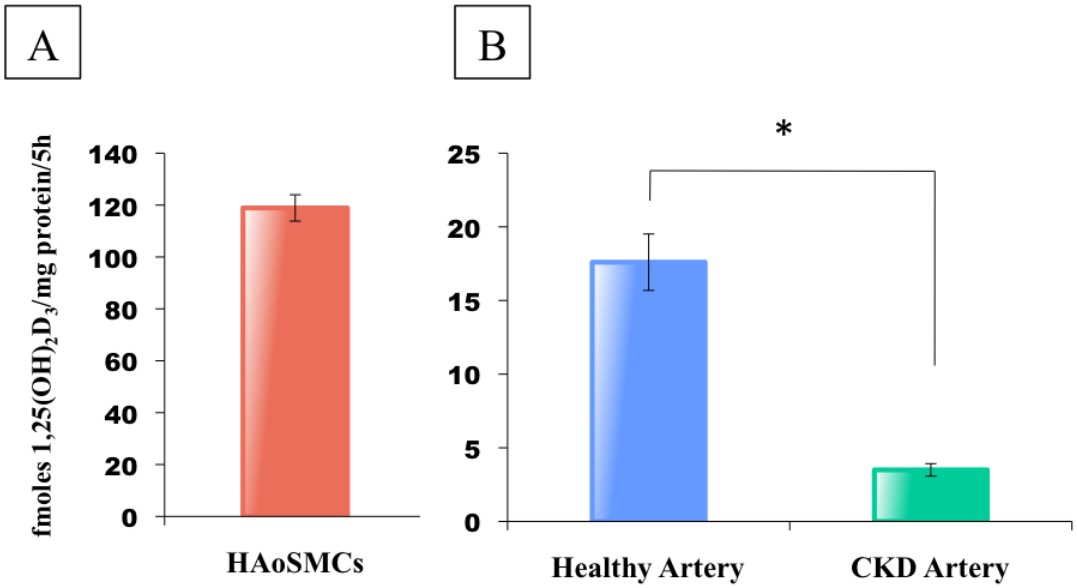


***Figure 4.15 Synthesis of 1,25(OH)$_2$D$_3$ in Human Aortic Smooth Muscle Cells (HAoSMCs), Healthy Artery and Chronic Kidney Disease (CKD) Artery****, as determined by EIA; (A)HAoSMCs (B) Arteries; data represent the mean ± SEM, healthy n=8, CKD=11, *p<0.05 as determined by Student's t-test. Note different scales.*

## 4.3 Discussion

Experimental evidence presented in this chapter strongly suggests that vasculature is a target tissue for $1,25(OH)_2D_3$, as confirmed by the increased expression of *VDR* mRNA and protein resulting from the treatment. 10 nM $1,25(OH)_2D_3$ has been shown in the past to significantly decrease DNA synthesis, in an *in vitro* model of human VSMCs (Somjen *et al.* 2005), however as it is extremely difficult to measure local concentrations of $1,25(OH)_2D_3$ elsewhere than in plasma, the treatment concentrations of $1,25(OH)_2D_3$ used in studying local changes in cell and tissue physiology, have been based on previous experimental work of others. 100 nM $25(OH)D_3$ has been previously shown to induce optimal cellular $25(OH)D_3$ to $1,25(OH)_2D_3$ conversion rate of 4-10%, in osteoblasts (Atkins *et al.* 2007).

My studies of vitamin D system in HAoSMCs indicated that exogenous 10 nM $1,25(OH)_2D_3$ induces responses similar to the ones seen in kidney proximal tubular cells. Excess of free and available $1,25(OH)_2D_3$ induced VDR expression in dose dependent manner. Increased rate of VDR/$1,25(OH)_2D_3$ complex formation consequently suppressed *CYP27B1* mRNA and protein as early as at 6 hours concomitantly inducing astonishing response in *CYP24A1* mRNA and protein, to counteract the effect of $1,25(OH)_2D_3$/VDR complex formation. On this basis it is not unreasonable to state that the vascular vitamin D system is governed by the same classical negative feedback regulation as the one in the endocrine system. An interesting observation was that 10 nM $1,25(OH)_2D_3$ at 48 hours did not alter 1α-OHase and 24-OHase protein, which implied that the clearance of $1,25(OH)_2D_3$ in HAoSMCs must be very effective. This phenomenon most probably would not be observed had the $1,25(OH)_2D_3$ been continually repleted. This mechanism makes vascular responses very dynamic and protects cells from excess of $1,25(OH)_2D_3$. It

has been postulated that $25(OH)D_3$ can also bind to VDR, but with much lesser affinity (Eisman and DeLuca 1977). In my study 100 nM $25(OH)D_3$ appeared to almost mimic the changes in *VDR, CYP27B1* and *CYP24A1* mRNA induced in HAoSMCs by $1,25(OH)_2D_3$ (10 nM). Interestingly the key difference between 10 nM $1,25(OH)_2D_3$ and 100 nM $25(OH)D_3$ was the effect of each on 1α-OHase protein expression. The first one rapidly (by 6 hours) inhibited the synthesis of 1α-OHase (result of abundance of active vitamin D), whereas the latter induced 1α-OHase protein expression (by 24 hours; result of abundance of substrate for 1α-hydroxylation). Highest concentrations of $1,25(OH)_2D_3$ (100 nM) or $25(OH)D_3$ (1000 nM) had different effect on *CYP24A1* mRNA expression. This may be due to the build up of different catabolites – $1,24,15(OH)_3D_3$, which is inactive and does not inhibit *CYP24A1* mRNA synthesis (accumulation of which may be an effect of $1,25(OH)_2D_3$ abundance), or $24,25(OH)_2D_3$, which may exhibit enough activity analogous to $1,25(OH)_2D_3$, suppressing *CYP24A1* mRNA synthesis (accumulation of which may be an effect of $25(OH)D_3$ abundance). Another factor may be biological effect of $25(OH)D_3$ directly competing with $1,25(OH)_2D_3$ for VDR.

Originally, $1,25(OH)_2D_3$ synthesis was described in kidney (Fraser DR; Kodicek, E. 1970, Midgett *et al.* 1973). Unfortunately, to date only limited cell models of the normal human kidney exist: HKC-8 from proximal tubule cells (Racusen *et al.* 1997) and the HCD, distal convoluted tubule cells (Prie *et al.* 1995). The vitamin D system has been well characterized in these cells (Bland *et al.* 1999, Bland *et al.* 2001). Evidence suggests that renal production of $1,25(OH)_2D_3$ is regulated differently to some extra-renal sites. As described Chapter 1.4, in kidney synthesis of $1,25(OH)_2D_3$ is enhanced by increases in the serum PTH concentration, a drop in serum phosphate concentration, and a decrease in the activity of the competing 24-OHase. To date, the

most extensively studied extra-renal model of vitamin D system, after colon, prostate, decidua, placenta, bone and breast tissue, are human macrophages, and therefore will be used for simple comparisons. It is important to note that macrophages are cells of the immune system and their role differs vastly from that of VSMCs or kidney cells, which consequently highlights the potential variety of vitamin D tissue-specific actions. Studies in patients with sarcoidosis (a condition characterized by increased intestinal calcium absorption and bone resorption, where common symptoms include hypercalcaemia, ultimately resembling $1,25(OH)_2D_3$ intoxication) demonstrated high levels of 1α-OHase in macrophages isolated from the lymph nodes, which was not inhibited by treatment with $1,25(OH)_2D_3$, *in vitro* (Hewison *et al.* 1989, Reichel *et al.* 1987). Both renal and macrophage 1α-OHase are products of the same gene (Smith S. J. *et al.* 1999) (existence of a number of non-coding functionally active splice variants of *CYP27B1* has been demonstrated in HKC-8 cells (Wu *et al.* 2007)), activity of both is dependent on the availability of nitric oxide, both require the presence of a flavoprotein, ferredoxin reductase, an electron source and a molecular oxygen in order to attach an oxygen atom to the substrate (Shany *et al.* 1993), both are inhibited by specific compounds which compete for electrons or oxygen (Adams *et al.* 1994), both require secosterol for a substrate (Reichel *et al.* 1987) and both exhibit highest affinity for 25(OH)D, then $24,25(OH)_2D$ (Fraser D. R. 1980, Hewison M. 2011). Interestingly though, macrophages have no $1,25(OH)_2D$-directed 24-OHase activity, as opposed to renal epithelial cells, where higher levels of 24-OHase can block 25(OH)D or $1,25(OH)_2D$ down the catabolic 24-OHase pathway. A possible explanation may be the existence of 24-OHase SV (splice variant), which due to specific truncation cannot be targeted to mitochondrial membrane, lacks catabolic activity and is highly abundant in

cytoplasm, potentially readily binding to 25(OH)D and $1,25(OH)_2D$ (Adams *et al.* 2007, Hewison M. 2011). In vasculature, basal levels of *CYP24A1* mRNA are higher than those of *CYP27B1*, and for such system to maintain stable levels of $1,25(OH)_2D_3$, 1α-OHase must have higher affinity for $25(OH)D_3$ than 24-OHase, as described in kidney (Henry H. L. 1979). Regardless of this, 24-OHase through its ability to reach high concentrations has a high capacity for acting on $25(OH)D_3$, taking into consideration how readily 24-OHase levels can be upregulated by increased $1,25(OH)_2D_3$ (Hewison M. 2011, Omdahl J. L. *et al.* 2001). Interestingly, evidence from studies on adenine induced CKD rats demonstrated, that as $25(OH)D_3$ and $1,25(OH)_2D_3$ levels decrease, *CYP24A1* mRNA and protein are strongly induced and *CYP27B1* mRNA and protein levels do not change (Anderson *et al.* 2003).

To recognise the role for extra-renal vitamin D system metabolism and regulation, the ground-braking experiments of Dusso and colleagues from over two decades ago must be considered (Dusso *et al.* 1988, Dusso *et al.* 1991). In these neat studies, supplementation of anephric haemodialysed patients with $25(OH)_2D_3$ resulted in significantly elevated $1,25(OH)_2D_3$ levels in the serum, indicating that this enhanced net synthesis of $1,25(OH)_2$ must have been attributed to extra-renal sites. What is more, no significant changes in serum calcium, i-PTH nor phosphate levels were recorded, confirming that the function of the vitamin D system in extra-renal sites may vary from the one in kidney. So the question to be asked is what causes the impaired serum $25(OH)D_3$ availability for renal and extra-renal $1,25(OH)_2D_3$ synthesis and is this relevant to autocrine VDR activation involved in cardiovascular protection? Exposure of normal human arteries to the exogenous 10 nM $1,25(OH)_2D_3$ resulted in the same responses as the ones in our primary cell *in vitro*

model system. Most significant changes occurred at 48 hours. Importantly, real-time RT-PCR analyses and Western analyses confirmed that the expression of *VDR*, *CYP27B1* and *CYP24A1* mRNA were altered in CKD vessels, however these changes have not exactly mirrored the changes in protein described in the previous chapter. The main distinction was 1α-OHase, which was significantly reduced at protein level in vessels from CKD patients and significantly elevated at mRNA level. The fact that RNA levels are not reflected in protein may indicate dysregulation of the system. Interestingly, macrophages exhibit very low basal 24-OHase activity, as opposed that in renal epithelial cells. Activity of 24-OHase in VSMCs has not been investigated to date, although based on our experimental work it became evident, that basal *CYP24A1* mRNA levels are higher than these of *VDR* and *CYP27B1* mRNA levels. Previous studies by others have shown that in both systems: macrophages and renal epithelial cells, treatment with exogenous $1,25(OH)_2D$ leads to potent increase in *CYP24A1* mRNA production. Again, one of the explanations could be presence of 24-OHase-SV. Another plausible rationalization could be action of siRNA species or any other factors ultimately involved in undesired post-transcriptional modifications of *CYP27B1* mRNA, as well as those interfering with protein translation, in VSMCs. What is more, a challenge with 10 nM $1,25(OH)_2D_3$ in uraemic arteries had no inhibitory effect on *CYP27B1* mRNA levels and indeed induced significant upregulation of *CYP24A1* – a result similar to the one described by Anderson in CKD rats. The 24-OHase protein induction was significantly higher in CKD compared to the one in healthy arteries. This on the other hand, seems to translate into our earlier observations, where *CYP24A1* was increased in CKD vessels (Chapter 3). Notably, increased expression of 24-OHase in these ureamic tissues may attenuate local levels of $1,25(OH)_2D_3$ either by converting the active metabolite to

$1,24,25(OH)_3D_3$ or by competing with 1α-OHase for a substrate ($25(OH)D_3$), generating $24,25(OH)_2D_3$. It is important to note, that the astonishing inhibitory ratio of *CYP24A1* to *CYP27B1* mRNA of 1: 1600 calculated based on the Real Time RT-PCR results, ought to be interpreted with caution as there are limits to translating RNA levels into activity due to possible action of micro RNAs preventing proportion of the *CYP24A1* mRNAs form being translated into fully functional enzymes.

Evidence suggests that $24,25(OH)_2D$ may have an effect on bone mass and metabolism. It has been reported that in hyperphosphataemic mice it normalized bone resorption (Ono *et al.* 1996). More recently, increased serum $24,25(OH)_2D_3$ (presumably due to increased 24-OHase activity) has been reported in children with severe osteogenesis imperfecta, a primary bone disorder, where bone mass is reduced leading to extremely fragile bones (Edouard *et al.* 2012). It can be speculated that the mechanism, which can be protective in bone, may lead to uncoupling between 1α-OHase, 24-OHase and VDR in other tissues, with VSMCs being an example. In Chapter 3, 24-OHase protein not only was shown to be increased in CKD arteries, compared to controls, but also increased 24-OHase protein expression co-localised with areas of calcification, RUNX-2 and sclerostin protein expression. On the basis of results presented in this chapter, it is plausible that when cells have higher than normal 25(OH)D : $1,25(OH)_2D_3$ ratio, 24-OHase may be competing for a substrate, by hydroxylating both $1,25(OH)_2D_3$ and $25(OH)D_3$, It would appear that a presumptive build up of $24,25(OH)_2D_3$ inhibited *CYP24A1* mRNA expression in VSMCs. It is possible that in CKD, this 24-OHase regulating mechanism is not functioning properly, leading to increased *CYP24A1* mRNA and protein synthesis. Or increased 24-OHase in CKD aggravates substrate deprivation, by competing with 1α-OHase for $25(OH)D_3$.

Decreased *VDR* mRNA and protein in the examined CKD vessels may further explain no significant inhibition in the transcription of *CYP27B1* mRNA and consequently high *CYP24A1* levels. Interestingly, there might be another cellular factor either contributing to accumulation of 1α-OHase at a pre-translation stage or a factor, which is controlling 1α-OHase protein degradation in CKD in order to explain our observations. High basal levels of *CYP27B1* mRNA have been documented in hyperplastic human parathyroid glands secondary to CKD, as well as in parathyroid and breast carcinomas (Segersten *et al.* 2002, Segersten *et al.* 2005). 10 out of 15 parathyroid adenoma and 8 out of 10 hyperplastic parathyroids from uremic patients had more than a 2-fold increase of *CYP27B1* mRNA content compared to normal parathyroid glands. Interestingly, a subset of both adenomatous and hyperplastic glands showed reduced 1α-OHase expression. It is important to note that the potential effects of variable serum DBP on total versus free vitamin D metabolite levels are an important consideration in examining associations between these metabolites in CKD or any other pathology. Another factor influencing abnormal expression of the arterial vitamin D system in CKD may be a tissue specific uptake of vitamin D metabolites.

Detection of $1,25(OH)_2D_3$ synthesis in both HAoSMCs and human arteries, suggested 1α-OHase activity and confirmed the presence of a functional intact vitamin D system in vasculature. The significantly higher synthesis of $1,25(OH)_2D_3$ per milligram of protein in HAoSMCs compared to CKD arteries might have been attributed to the abundance of not only SMC protein, but also those from other cell types present in the artery. It could be speculated that the VSMCs are the predominant producers of $1,25(OH)_2D_3$ in the human vasculature. Decreased expression of the 1α-OHase protein in CKD arteries (Chapter 3) is also reflected in

significantly decreased synthesis of $1,25(OH)_2D_3$, suggesting that 1α-OHase activity may be essential for the maintenance of vascular health,

In summary, using an *in vitro* model of HAoSMCs and normal human arteries, the presence of a functioning, intact VDR signalling was demonstrated. Data showed that the regulation of the VDR metabolism in these systems appeared to be similar to the one described by others in proximal tubular cells of the kidney (since challenge of HAoSMCs with $1,25(OH)_2D_3$ significantly inhibited *CYP27B1* mRNA and protein expression) and dissimilar to the vitamin D metabolism is macrophages. What is more, it was demonstrated that local 1α-OHase activity allowed VSMCs to synthesize $1,25(OH)_2D_3$ from $25(OH)D_3$. This brings to attention the importance of $25(OH)D_3$ sufficiency, i.e. availability of the substrate for local actions. Lastly, the data revealed significant changes in expression of *VDR*, *CYP27B1* and *CYP24A1* on both mRNA and protein level between healthy and CKD arteries. Further to that, it became evident that these changes may trigger abnormal responses to exogenous $1,25(OH)_2D_3$, especially by increasing 24-OHase synthesis. Consequently, from the clinical perspective, prolonged treatment with $1,25(OH)_2D_3$ and possibly other active vitamin D compounds may result in activation of 24-OHase, potentially causing direct vascular therapeutic resistance. High and dynamic regulation of the vascular vitamin D system may be an important feature, which if utilized correctly could prevent vascular CKD complications.

# Chapter 5

# Classical Regulators of the Vitamin D System: Calcium, Phosphate and Inflammatory Cytokines

## 5.1 Background

Calcium and phosphate are classical regulators of the endocrine vitamin D system where they control of 1α-OHase and 24-OHase in kidney (Hughes *et al.* 1975, Zehnder *et al.* 2008). Inflammatory cytokines on the other hand, are classical regulators of the local auto/paracrine vitamin D system. Nonetheless, there is growing evidence that calcium, phosphate, TNF-α and INF-γ play an important role in regulating both systemic and local $1,25(OH)_2D$ metabolism.

The renal 1α-OHase is induced by low dietary calcium both directly and through increased PTH/cAMP and is suppressed by high dietary phosphate, through the action of FGF-23 (Bland *et al.* 1999, Hughes *et al.* 1975, Omdahl J. L. *et al.* 1972, Shimada *et al.* 2004b, Tanaka and Deluca 1973). Recent evidence shows, that while circulating $1,25(OH)_2D_3$ produced by the kidney regulates systemic calcium and phosphate balance through actions on the intestine, parathyroid glands, bone and kidney, it appears that $1,25(OH)_2D_3$ produced locally in the parathyroid glands does

not exert calcitropic actions (Bikle D. 2009). What is more, Ritter and colleagues have demonstrated that high concentrations of calcium significantly upregulate *CYP27B1* and *CYP24A1* mRNA expression and activity of both enzymes in cultured hyperplastic human parathyroid cells (Ritter *et al.* 2012). Previous reports indicated similar results in bone (Anderson *et al.* 2010). This is directly opposite to the effect observed in kidney. The current literature on the implications of this sophisticated regulation of local vitamin D metabolism by calcium and phosphate in other extra-renal tissues expressing 1α-OHase activity, especially human vasculature is limited.

In vasculature, intracellular calcium is an important regulator of the vascular tone and blood flow (Green *et al.* 2006), whereas extracellular calcium sensing may play a role in maintenance of vascular health. Evidence shows that the calcium sensing receptor (CaSR) is not only expressed in human artery and VSMCs, but also that its expression is significantly lower in patients with ESRD (Molostvov *et al.* 2009, Molostvov *et al.* 2007). Studies by Schroff and co-workers have further demonstrated that CKD vessels, *ex vivo*, exposed to high calcium and/or high phosphate concentrations accumulated calcium, as opposed to normal vessels. High calcium and high phosphate have been shown to increase calcification independently through induction of apoptosis and excretion of mineralising vesicles (Reynolds *et al.* 2004, Shroff R. C. *et al.* 2010). There is growing evidence that the systemic regulation of mineral homeostasis may be fine-tuned by local 1α-OHase in organs such as bone, vasculature and parathyroid.

As well as mineral and hormonal dysregulation, CKD is a state of mild chronic inflammation. Complex interactions between infiltrating immune cells and vascular cells result in a production of cytokines and growth factors (Geng *et al.* 1997). Local 1α-hydroxylation may be an important mechanism by which VSMCs can reach the

appropriate levels of $1,25(OH)_2D$ needed to shape and control cellular proliferation, differentiation, apoptosis an combat inflammation, without affecting systemic levels of the hormone.

The proinflammatory cytokine TNF-α in particular seems to be an important player in VC, due to its suppressive action on VDR activation in SMCs of human bronchi (Agrawal *et al.* 2012). Furthermore it plays a role in an enhancement of osteoblastic transdifferenciation of human cardiovascular cells by increasing the transcription of bone-specific genes, such as RUNX-2, osteocalcin and ALP (Agrawal *et al.* 2012, Tintut *et al.* 2000). INF-γ, on the other hand inhibits the synthesis of α-SM actin, which attenuates cellular proliferation (Hansson *et al.* 1989). Synergistically, TNF-α and INF-γ have been shown to induce SMCs death (Geng *et al.* 1996). IL-6 is a pro-inflammatory cytokine, synthesis of which is induced by TNF-α and a synergistic action of IL-17A and INF-γ (Eid *et al.* 2009).

Taken together, evidence from previous studies demonstrating that calcium, phosphate, TNF-α, INF-γ, IL-6 and IL-17A independently affect non-renal metabolism of $1,25(OH)_2D$, in combination with my work described in chapter 4, which has demonstrated a functional, self-regulatable vascular vitamin D system, led to the assumption that there may be a specific role for all of these factors in regulating the vitamin D system locally in vascular cells in an autocrine fashion.

This chapter examines the effect of calcium or phosphate on the expression of VDR, 1α-OHase and 24-OHase in primary cultures of HAoSMCs. In addition, the outcome of treatment of HAoSMCs with the inflammatory cytokines, such as TNF-α, INF-γ, IL-6 and IL-17A on local vitamin D metabolism in these cells was examined.

## 5.2 Results

### 5.2.1 Regulation of the Vascular Smooth Muscle Cell Vitamin D System by Calcium

HAoSMCs were grown in DMEM/F-12 in the absence of serum for 24 hours prior to culture in media containing 1 mM calcium (basal concentration in DMEM/F-12 and the normal physiological concentration of calcium in ionised form) or media containing 2 mM or 3 mM of calcium for 6, 24 and 48 hours.

#### *5.2.1.1 Vitamin D Receptor is Modulated by Calcium*

2 mM calcium increased VDR protein expression at 6 and at 24 hours, but the increase was not quite statistically significant *($p= 0.068$, $p= 0.067$* respectively; one-way ANOVA) the maximal effect observed at 24 hours *(Figure 5.1).* 3 mM calcium significantly elevated VDR protein expression by 2.6-fold as early as at 6 hours (*$p= 0.007$*) and 1.7-fold at 24 hours (*$p= 0.040$*), compared to the 1 mM control at corresponding time points *(Figure 5.1).*

#### *5.2.1.2 Calcium Induced an Early and Sustained Increase in 1α-OHase and 24-OHase*

2 mM calcium significantly increased 1α-OHase protein expression by 1.7-fold at 48 hours (*$p= 0.001$*) *(Figure 5.2).* Similarly, 3 mM calcium induced significant 2.3-fold increase in 1α-OHase expression at 6 hours (*$p= 0.009$*), 1.9-fold increase at 24 hours (*$p= 0.053$*) and 1.7-fold increase at 48 hours (*$p= 0.001$*).

24-OHase protein expression was significantly elevated by 7.4-fold at 6 hours in presence of 2 mM calcium *($p= 0.014$).* 24-hour and 48-hour incubation with 3 mM

calcium significantly induced 24-OHase protein expression 2.7-fold (*p= 0.033)* and 5.7-fold (*p= 0.037*) respectively, compared to the respective 1 mM 24-hour and 48-hour controls *(Figure 5.3).*

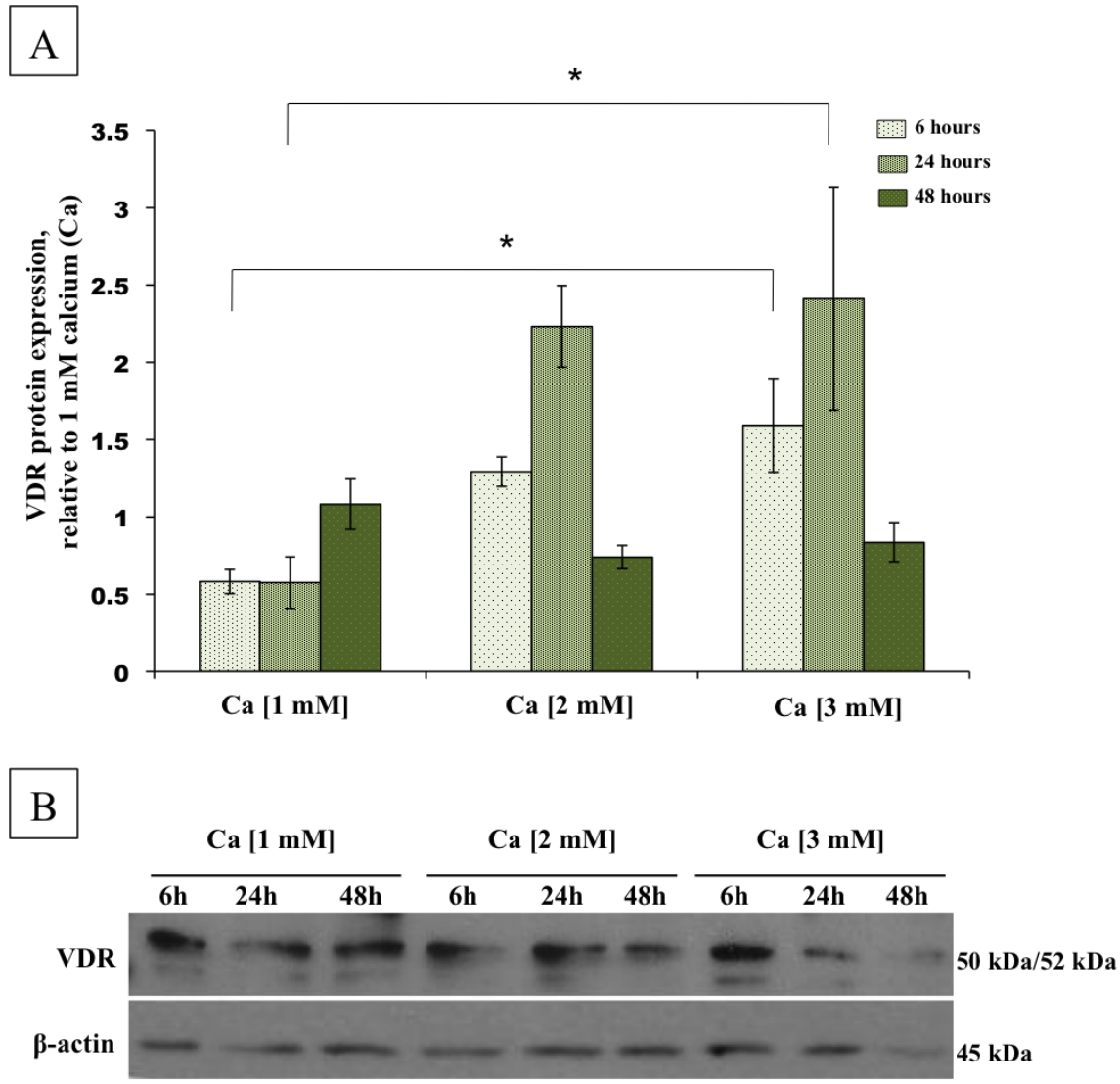


***Figure 5.1: The effect of Calcium on expression of the Vitamin D Receptor (VDR) protein in Human Aortic Smooth Muscle Cells (HAoSMCs) over time****. HAoSMCs were cultured in serum-free medium containing 1 mM calcium (normal) or 2 mM or 3 mM (high), for 6, 24 and 48 hours. (A) VDR protein expression normalised to β-actin protein levels, as demonstrated by Western blot analyses. Results represent mean ± SEM, n = 6, * p < 0.05; vs. 1 mM for each time point; data were analysed by one-way ANOVA. (B) Representative Western blot; cell lysates were separated by 10% SDS-PAGE and blotted with anti-VDR and anti-β-actin.*

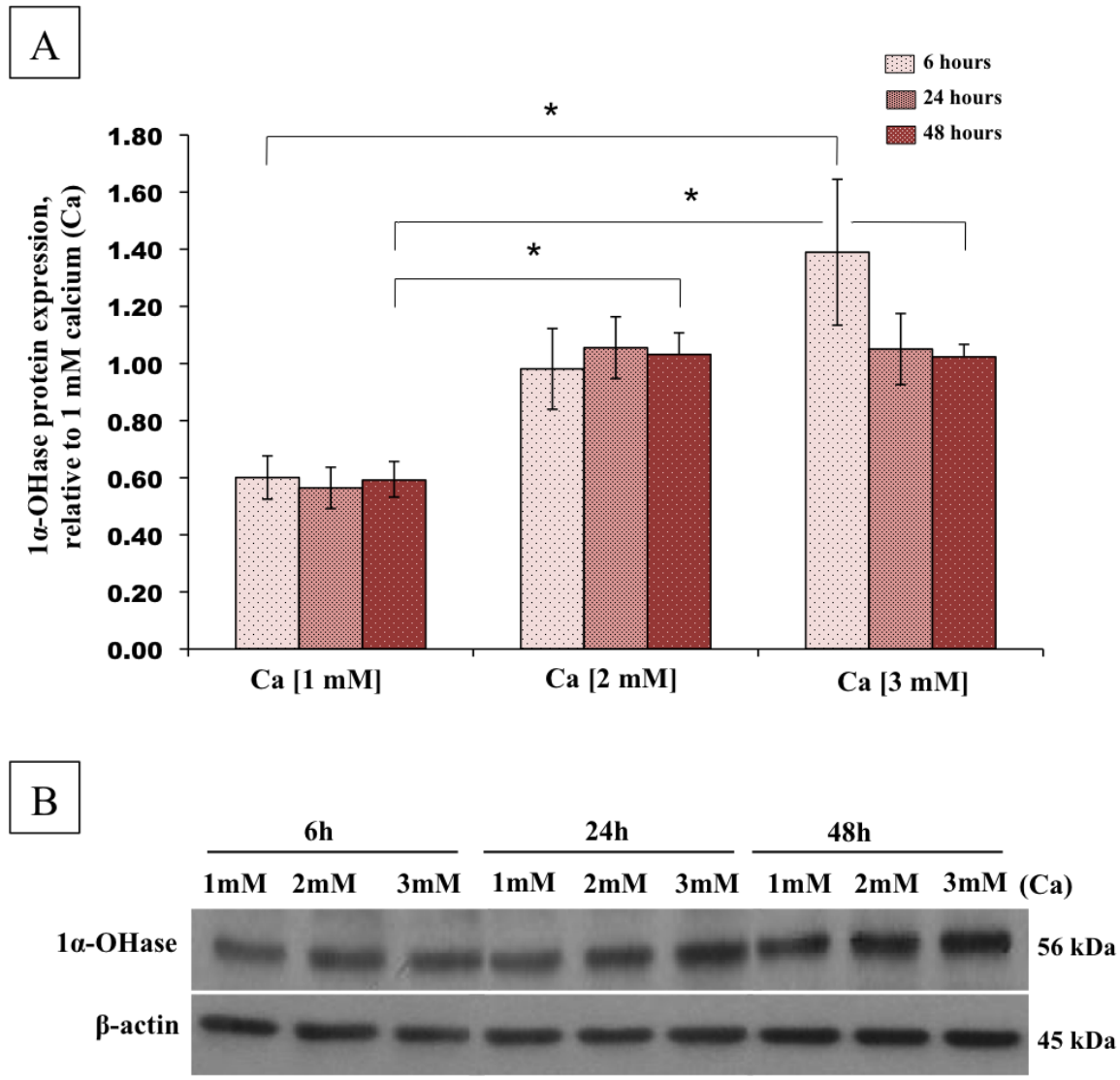


***Figure 5.2: The effect of Calcium on the 1α-Hydroxylase (1α-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *HAoSMCs were cultured in serum-free medium containing 1 mM calcium (normal) or 2 mM or 3 mM (high), for 6, 24 and 48 hours. (A) 1α-OHase protein expression, normalised to β-actin protein levels, as demonstrated by Western blot analyses. Results represent mean ± SEM, n = 6, * $p < 0.05$; vs. 1 mM for each time point; data were analysed by one-way ANOVA (B) Representative western blot - note different layout to the graph; cell lysates were separated by 10% SDS-PAGE and Western blotted with anti-1α-OHase and anti-β-actin.*

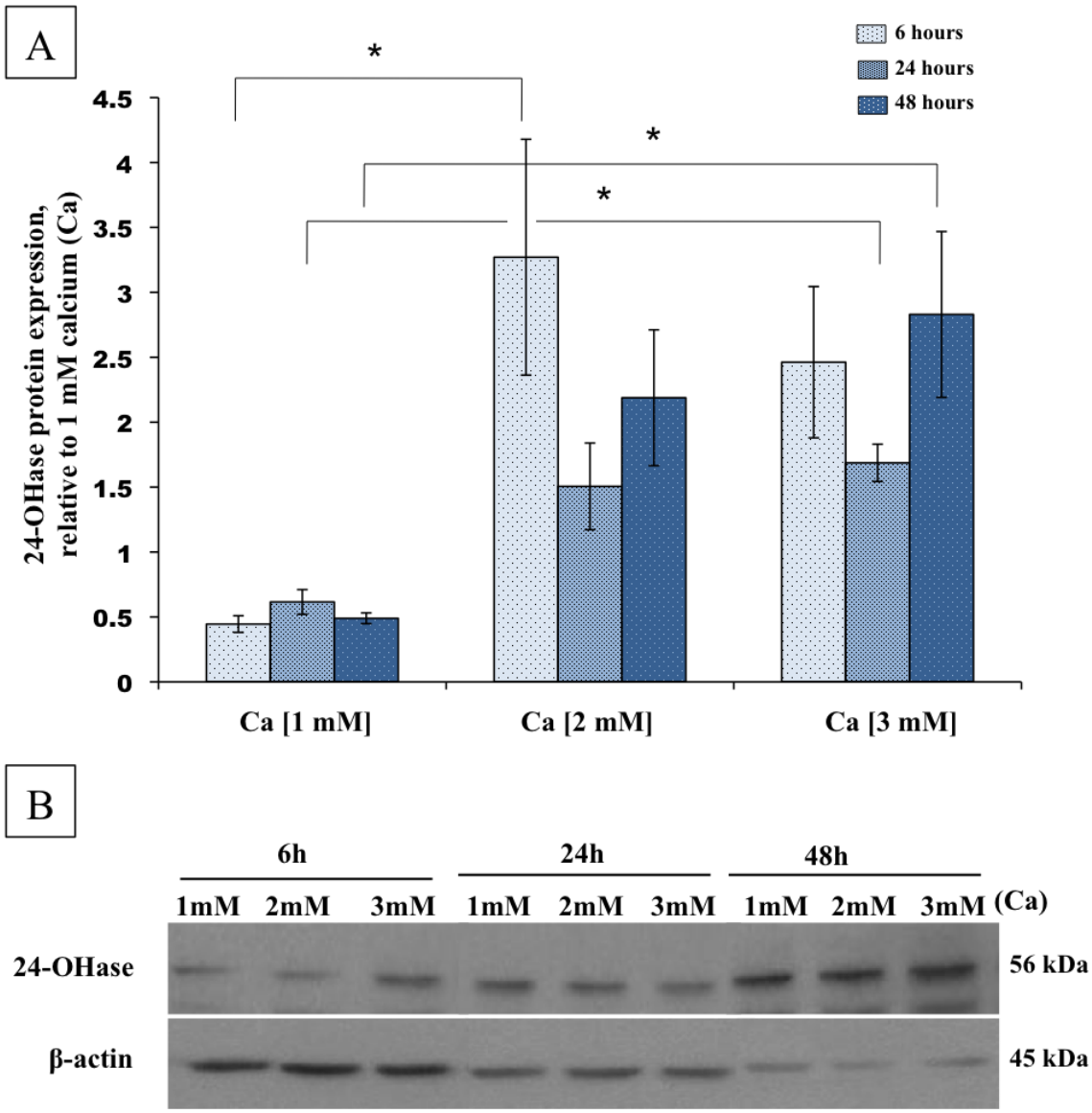


***Figure 5.3: The effect of Calcium on the 24-Hydroxylase (24-OHase) protein expression in (Human Aortic Smooth Muscle Cells) HAoSMCs over time****. HAoSMCs were cultured in serum-free medium containing 1 mM calcium (normal) or 2 mM or 3 mM (high), for 6, 24 and 48 hours. (A) 24-OHase protein expression, normalised to β-actin protein levels, as demonstrated by Western blot analyses. Results represent mean ± SEM, n = 6, * p < 0.05; vs.1 mM for each time point; data were analysed by one-way ANOVA. (B) Representative Western blot-note different layout to the graph; cell lysates were separated by 10% SDS-PAGE and Western blotted with anti-24-OHase and anti-β-actin.*

### 5.2.2 Regulation of the Vascular Smooth Muscle Vitamin D System by Phosphate

HAoSMCs were cultured in varying levels of phosphate (1,2 or 3 mM) for 6, 24 and 48 hours. The basal level of phosphate in the medium was 1 mM (normal physiological concentration is 0.5-1 mM in ionised form).

#### *5.2.2.1 Vitamin D Receptor is Not Affected by Phosphate*

2 mM phosphate had no effect on VDR protein expression *(Figure 5.4).*

#### *5.2.2.2 Phosphate Induces the 1α-OHase and 24-OHase Expression*

2 mM phosphate significantly induced 1α-OHase protein expression by 2.3-fold at 24 hours (*p= 0.044*) *(Figure 5.5),* 3 mM phosphate induced no significant change *(Figure 5.5).* 24-OHase protein was also induced by 2 mM phosphate, at 24 hours (3.2-fold, *p= 0.039*) and 48 hours (10-fold, *p= 0.005) (Figure 5.6).* 3 mM phosphate significantly induced 24-OHase protein expression by 6.6-fold by as early as at 6 hours (*p= 0.012*); at 48 hours 24-OHase protein levels were increased 12-fold (*p= 0.001),* compared to 1 mM phosphate at corresponding time points *(Figure 5.6).*

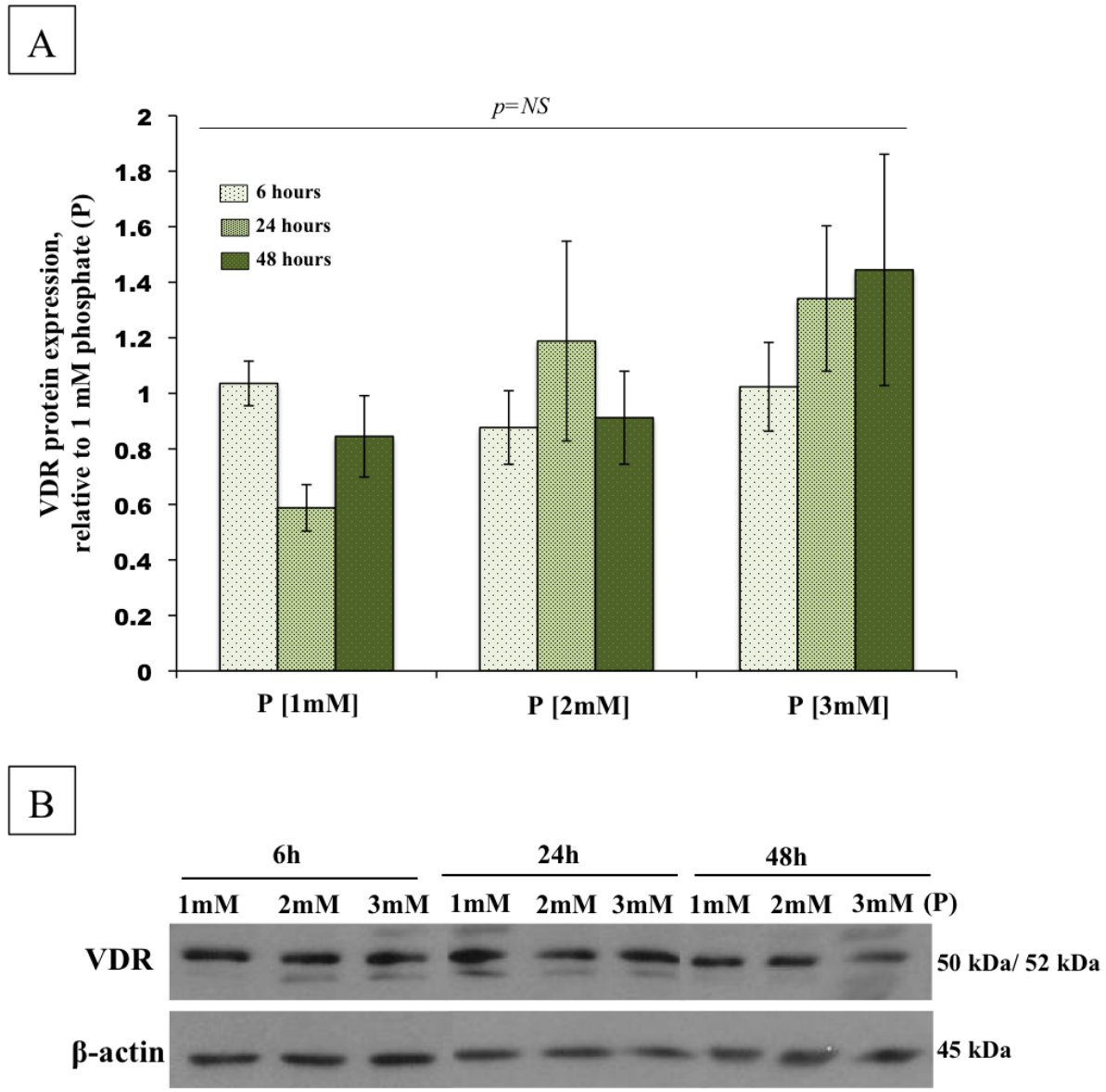


***Figure 5.4: The effect of Phosphate on the Vitamin D Receptor (VDR) protein expression in (Human Aortic Smooth Muscle Cells) HAoSMCs over time****. HAoSMCs were cultured in serum-free medium containing 1 mM phosphate (normal) or 2 mM or 3 mM (high), for 6, 24 and 48 hours. (A) VDR protein expression, normalised to β-actin protein levels, as demonstrated by Western blot analyses. Results represent mean ± SEM, n = 6, * p < 0.05; vs. 1 mM for each time point; data were analysed by one-way ANOVA. (B) Representative Western blot-note different grouping to the graph; cell lysates were separated by 10% SDS-PAGE and Western blotted with anti-VDR and anti-β-actin.*

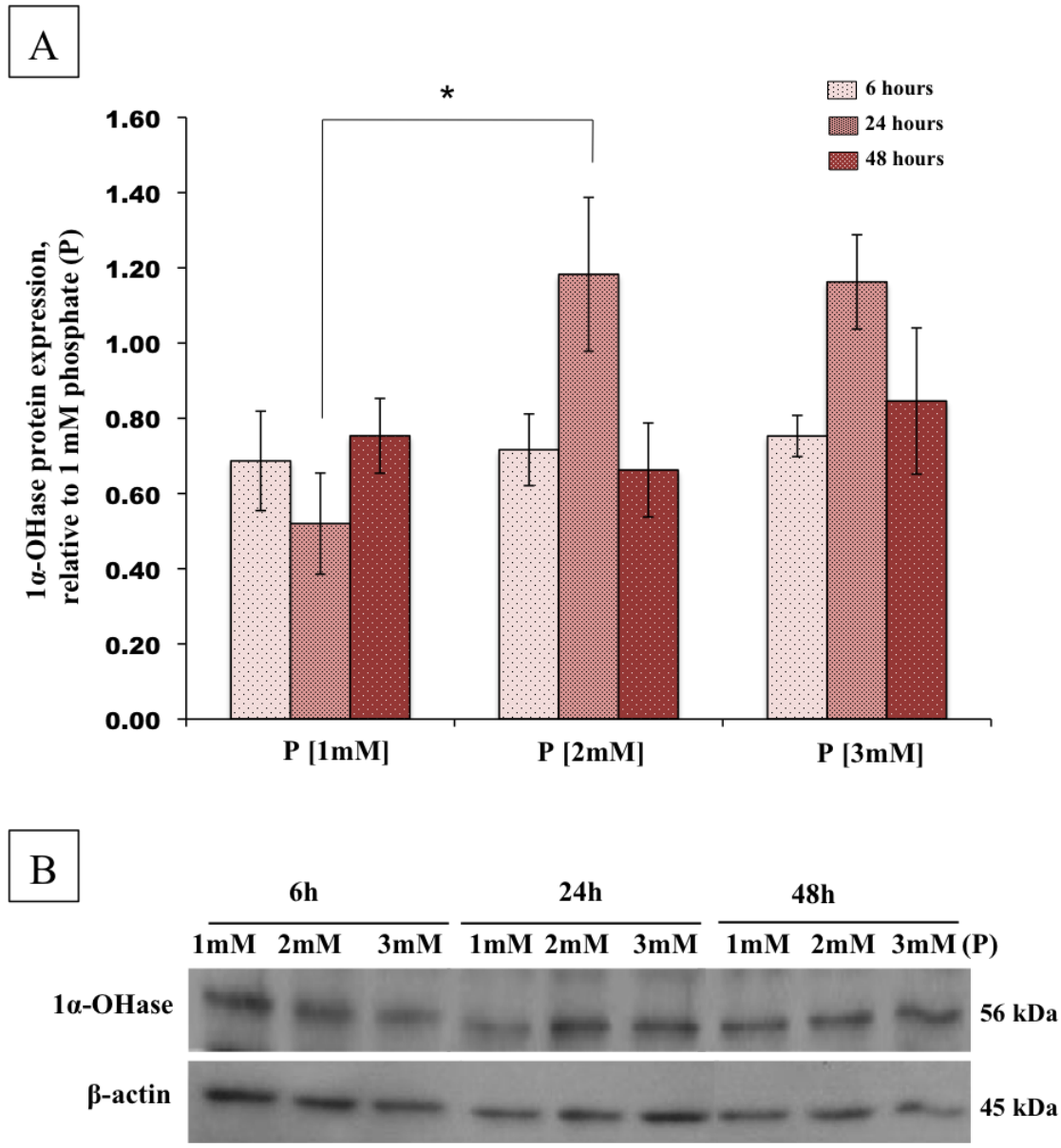


***Figure 5.5: The effect of Phosphate on the 1α-Hydroxylase (1α-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *HAoSMCs were cultured in serum-free medium containing 1 mM phosphate (normal) or 2 mM or 3 mM (high), for 6, 24 and 48 hours. (A) 1α-OHase protein expression normalised to β-actin, as demonstrated by Western blot analyses. Results represent mean ± SEM, n = 6, * p < 0.05; vs. 1 mM for each time point; data were analysed by one-way ANOVA. (B) Representative Western blot – note different layout to the graph; cell lysates were separated by 10% SDS-PAGE and Western blotted with anti-1α-OHase and anti-β-actin.*

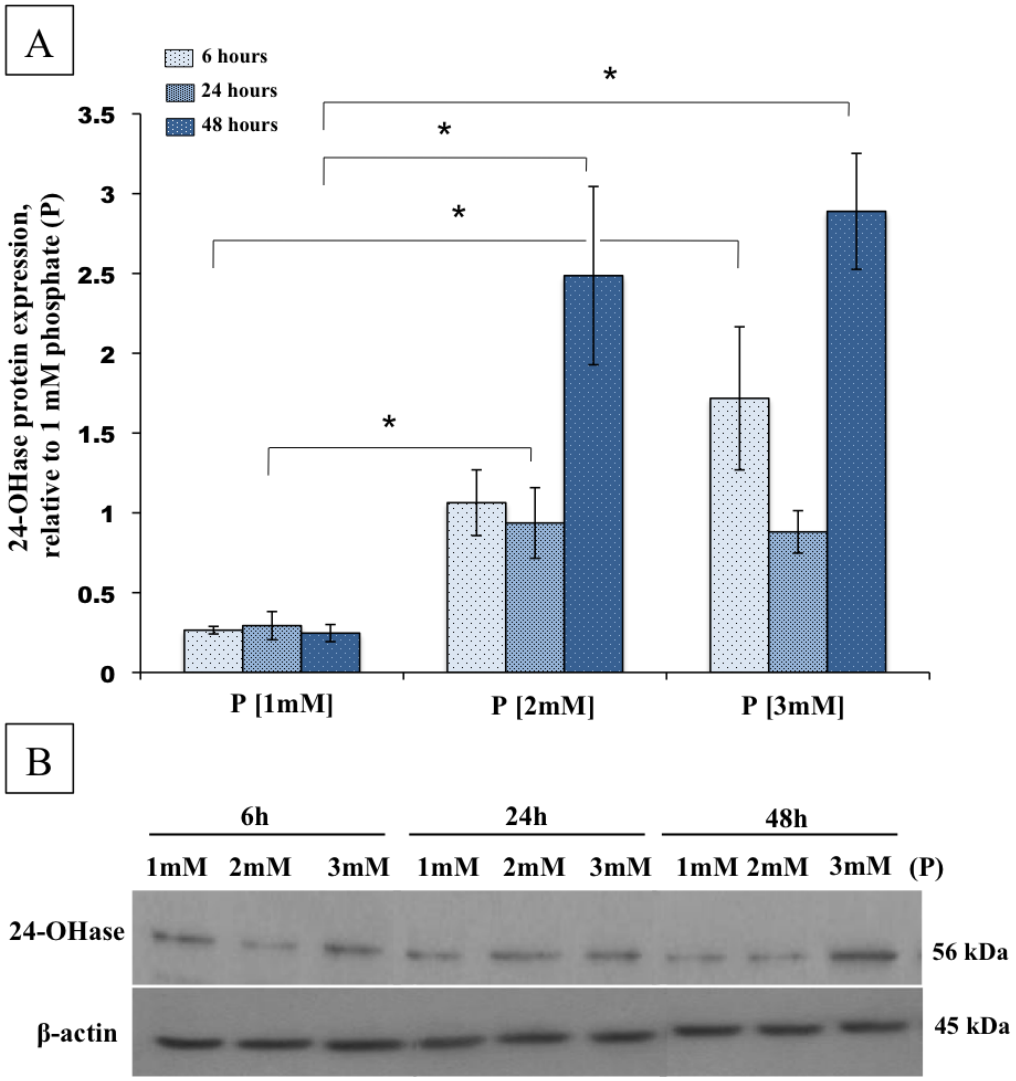


***Figure 5.6: The effect of Phosphate on the 24-Hydroxylase (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *HAoSMCs were cultured in serum-free medium containing 1 mM phosphate (normal) or 2 mM or 3 mM (high), for 6, 24 and 48 hours. (A) 24-OHase protein expression normalised to β-actin, as demonstrated by Western blot analyses. Results represent mean ± SEM, n = 6, * $p < 0.05$; vs. 1 mM for each time point; data were analysed by one-way ANOVA. (B) Representative Western blot – note different layout to the graph; cell lysates were separated by 10% SDS-PAGE and Western blotted with anti-24-OHase and anti-β-actin.*

### 5.2.3 The Effect of TNF-α on the Vitamin D System mRNA and Protein Expression in HAoSMCs and in both Healthy and CKD Arteries

The impact of TNF-α on local vitamin D metabolism, both in HAoSMCs and human arteries – in health and CKD were examined.

### *5.2.3.1 TNF-α Modulates the Vitamin D System in HAoSMCs*

In order to examine the effect of TNF-α on HAoSMCs vitamin D system both dose and time responses were investigated. Cells were treated with vehicle (PBS with 0.1% BSA) or 20 ng/ml TNF-α for 6 or 24 hours. The concentration 20 ng/ml was selected according to a prior dose response experiment, where HAoSMCs were treated with vehicle (PBS with 0.1% BSA), 1, 10 or 20 ng/ml TNF-α for 24 hours (Geng *et al.* 1996, Pryke *et al.* 1990, Stenvinkel *et al.* 2005). Both 1 and 20 ng/ml TNF-α appeared to upregulate VDR protein expression, but 20 ng/ml seemed to induce a stronger expression *(Figure 5.7A).* No changes were observed in 1α-OHase protein expression *(Figure 5.7B),* whereas 24-OHase protein appeared to be upregulated by 1, 10 and 20 ng/ml TNF-α *(Figure 5.7C).*

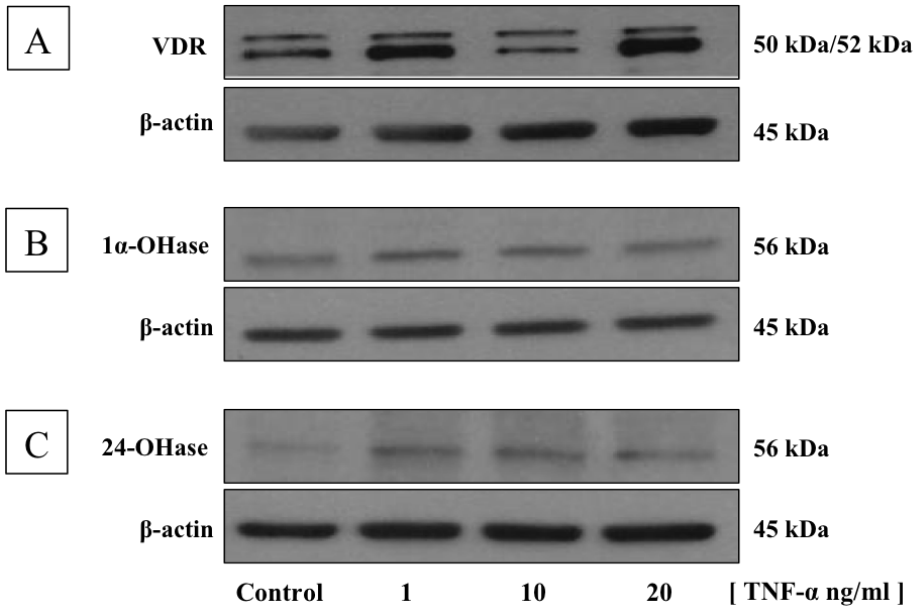


***Figure 5.7: TNF-α dose response in Human Aortic Smooth Muscle Cells (HAoSMCs), with effect on the Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) protein expression.*** *Cells were treated with vehicle (PBS with 0.1% BSA) or TNF-α (1, 10 or 20 ng/ml) for 24 hours. Representative Western blots: (A) VDR (B) 1α-OHase (C) 24-OHase, as demonstrated by Western blot analysis, β-actin for equal loading.*

TNF-α (20 ng/ml) had no effect on *VDR mRNA* expression *(Figure 5.8A).* Western blot analyses indicated that TNF-α (20 ng/ml) induced significant 1.8-fold increase in the VDR protein expression at 24 hours (*p= 0.013*) *(Figure 5.8B).*

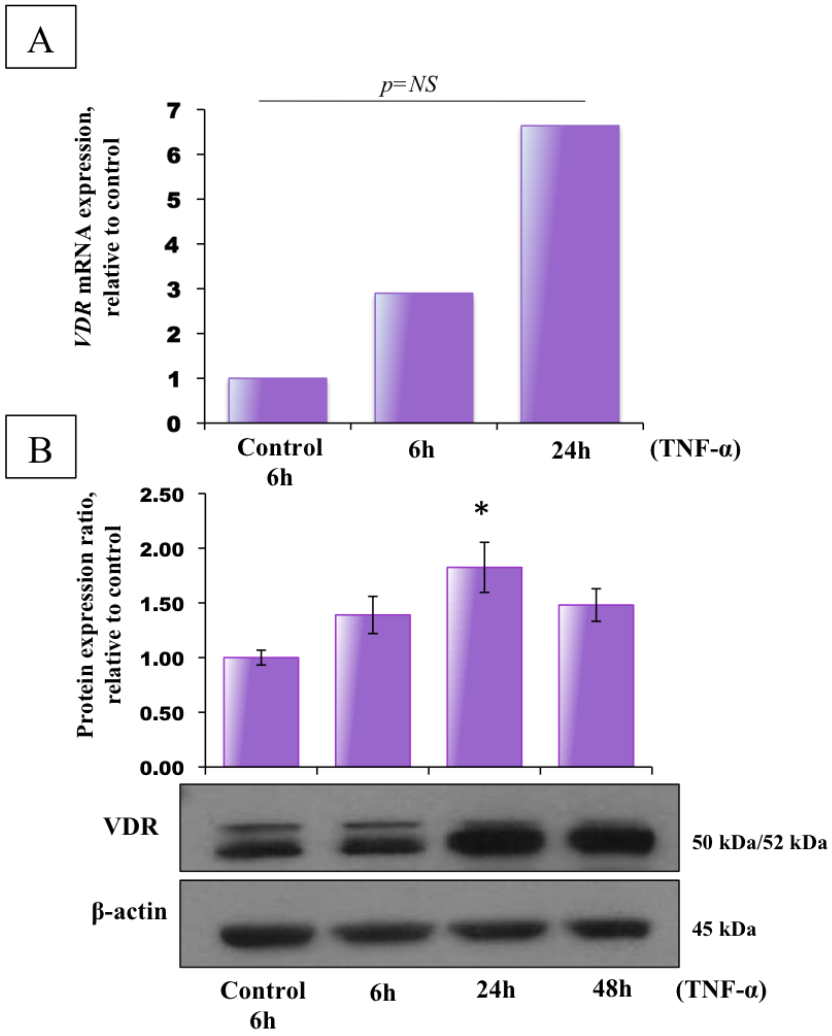


***Figure 5.8: The effect of TNF-α on the Vitamin D Receptor (VDR) mRNA and protein expression in HAoSMCs****. Cells were incubated with vehicle (PBS with 0.1% BSA) or TNF-α (20 ng/ml) for 6, 24 and 48 hours. (A) VDR mRNA expression normalised to 18S rRNA, as demonstrated by real time RT-PCR (B) VDR protein expression with representative Western blots, values normalised to β-actin protein levels, as demonstrated by Western blot analyses. Results represent mean±SEM, n = 6, * p < 0.05; vs. control, as determined by the one-way ANOVA.*

Furthermore, TNF-α had no effect on *CYP27B1* mRNA *(Figure 5.9A)* and *CYP24A1* mRNA *(Figure 5.9C).* TNF-α had no effect on 1α-OHase protein expression and caused a significant induction in 24-OHase protein at 6 hours *(Figure 5.9B).* An

increase in 1α-OHase activity induced by TNF-α has been observed previously in human umbilical vein endothelial cells (Zehnder *et al.* 2002b). 24-OHase protein expression was significantly induced by 50% at 6 hours ($p < 0.0001$) *(Figure 5.9D).*

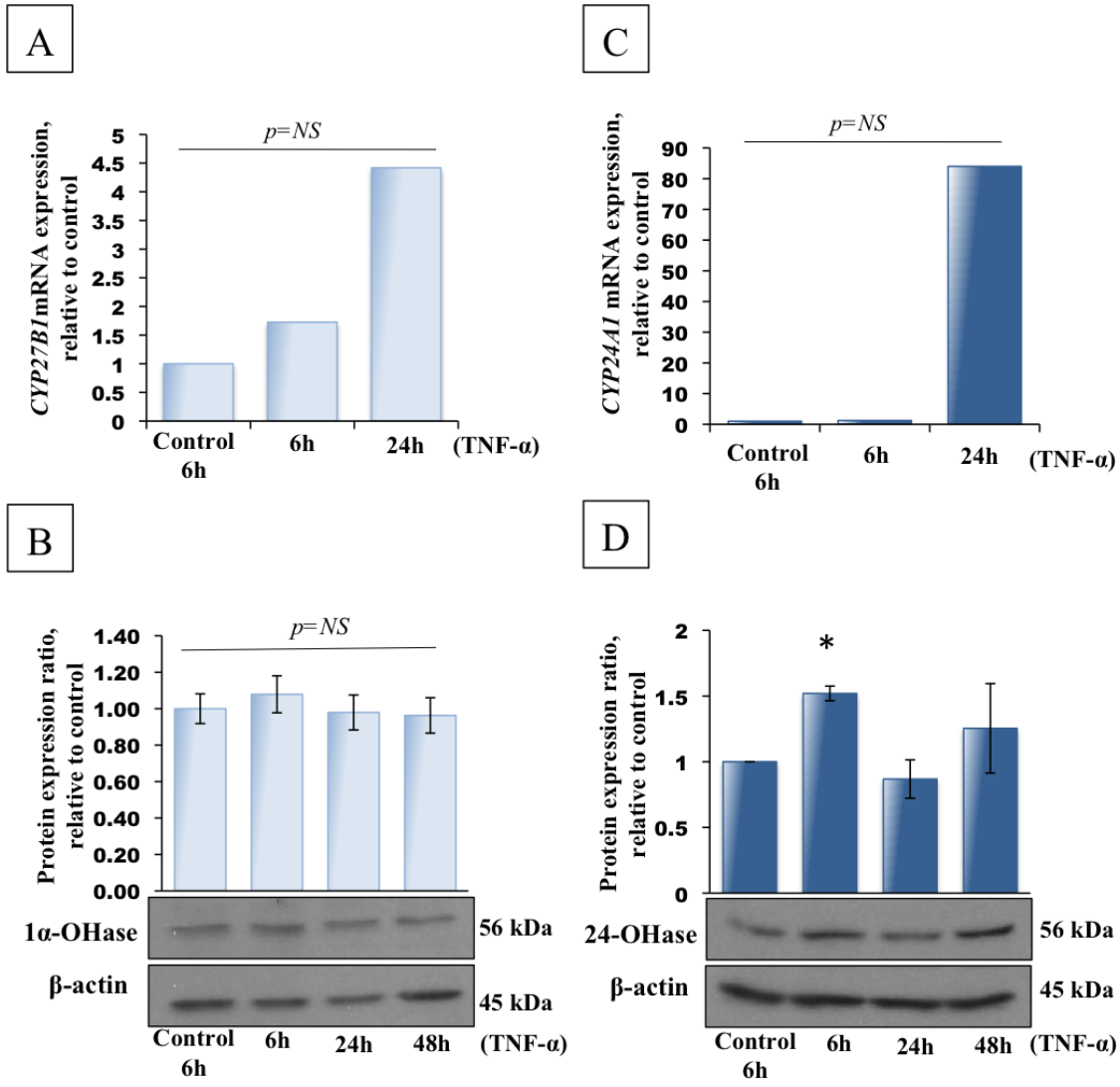


***Figure 5.9: The effect of TNF-α on the 1α-Hydroxylase (1α-OHase; CYP27B1) and 24-Hydroxylase (24-OHase; CYP24A1) protein and mRNA expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *Cells were incubated with vehicle (PBS with 0.1% BSA) or TNF-α (20 ng/ml) for 6, 24 and 48 hours. (A) CYP27B1 mRNA expression (B) 1α-OHase protein expression, with representative Western blots (C) CYP24A1 mRNA expression (D) 24-OHase protein expression, with representative Western blots. Protein expression was assessed by Western blot analyses and normalised to β-actin levels. Results represent mean±SEM, n = 6, * p <0.05; vs. control, as determined by one-way ANOVA. mRNA expression was assessed by real-time RT-PCR and normalised to 18S rRNA.*

#### *5.2.3.2 TNF-α Modulates the Vitamin D System in Human Arteries*

Arterial explants (renal and epigastric) from patients undergoing kidney transplantation (n = 11 healthy: donors; n = 16 CKD: recipients) were obtained with patients' informed consent (supplementary patient information *Table 3.1).* Vessels were prepared for treatment by removal of the surrounding fat and the connective tissue and by further washing and equilibration in DMEM/F12 medium, in absence of serum for 1 hour. Vessels were treated with vehicle (PBS with 0.1% BSA) or TNF-α (20 ng/ml) for 48 hours. Differences in mRNA expression were examined by real-time RT-PCR analysis, with values normalised to 18S rRNA levels.

*VDR* mRNA expression was lower in CKD arteries compared to healthy untreated controls. TNF-α significantly induced all three *VDR*, *CYP27B1* and *CYP24A1* mRNAs. The presence of TNF-α significantly induced *VDR* mRNA expression by 10-fold in healthy arteries ($p<0.001$), and 3-fold in CKD arteries ($p<0.001$) *(Figure 5.10A). CYP27B1* mRNA levels were two times higher in CKD arteries, compared with healthy controls (*Figure 5.10B*). TNF-α induced *CYP27B1* mRNA expression in healthy arteries by about 200% ($p<0.01$) and even greater induction (500%) was observed in CKD arteries ($p<0.001$) *(Figure 5.10B). CYP24A1* mRNA expression levels in healthy and CKD arteries were similar. 48-hour treatment with TNF-α caused a raise in *CYP24A1* mRNA, both in healthy (230%) ($p<0.001$) and CKD arteries (180%) ($p=0.001$), however the increase seen in CKD was not as high as the one observed in healthy arteries ($p=0.007$) *(Figure 5.10C).*

In order to examine the effect of $1,25(OH)_2D_3$ on inflammation, *IL-6* mRNA expression was assessed in presence of $1,25(OH)_2D_3$ and/or TNF-α in healthy human

arteries and CKD arteries. Arteries were incubated with vehicle (0.1% ethanol) or TNF-α (20 ng/ml) or $1,25(OH)_2D_3$ (100 nM) alone or together for 48 hours. *IL-6* mRNA levels were 54% lower in CKD arteries, compared to controls (*p=0.005*). Treatment with TNF-α massively induced *IL-6* mRNA in healthy arteries (1400%, $p<0.001$), and less in CKD (200%, *p<0.001*). $1,25(OH)_2D_3$ in the presence of TNF-α significantly inhibited *IL-6* mRNA induction ($p<0.001$ compared to TNF alone) (*Figure 5.11*).

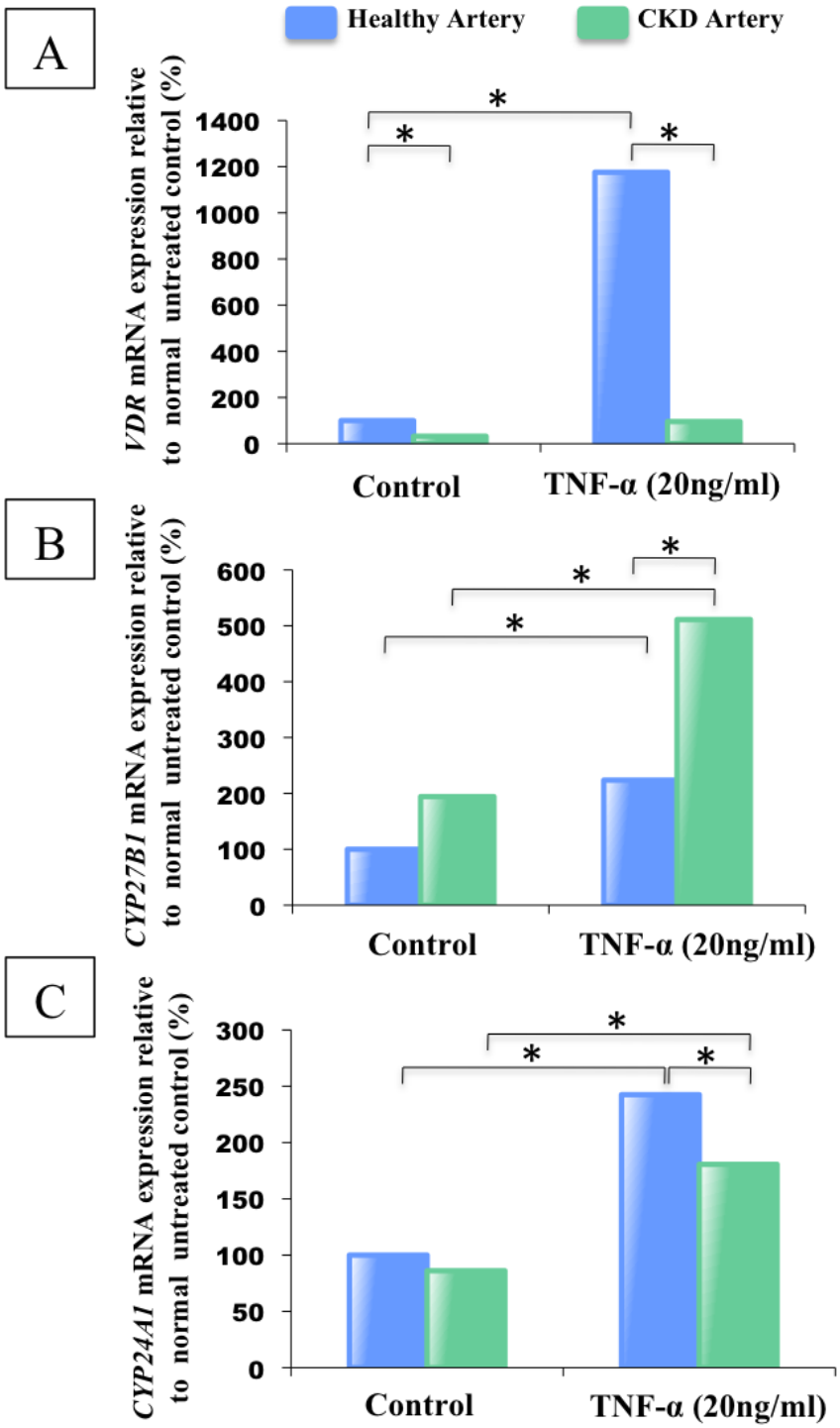


***Figure 5.10: The effect of TNF-α on the Vitamin D Receptor (VDR), 1α-Hydroxylase (CYP27B1) and 24-Hydroxylase (CYP24A1) mRNA expression in Healthy and Chronic Kidney Disease (CKD) arteries****. Arterial explants were incubated with vehicle (0.1% ethanol) or TNF-α (20 ng/ml) for 48 hours. Note the different scales (A) VDR mRNA expression, (B) CYP27B1 (C) CYP24A1 mRNA. Differences in mRNA expression were examined by real-time RT-PCR analyses (normalised to 18S rRNA levels). Results represent mean of relative mRNA levels; n =11 healthy, 16 CKD, * p < 0.05; vs. healthy untreated control and vs. healthy treated control; determined by one-way ANOVA. Data provided by Dr Guerman Molostvov.*

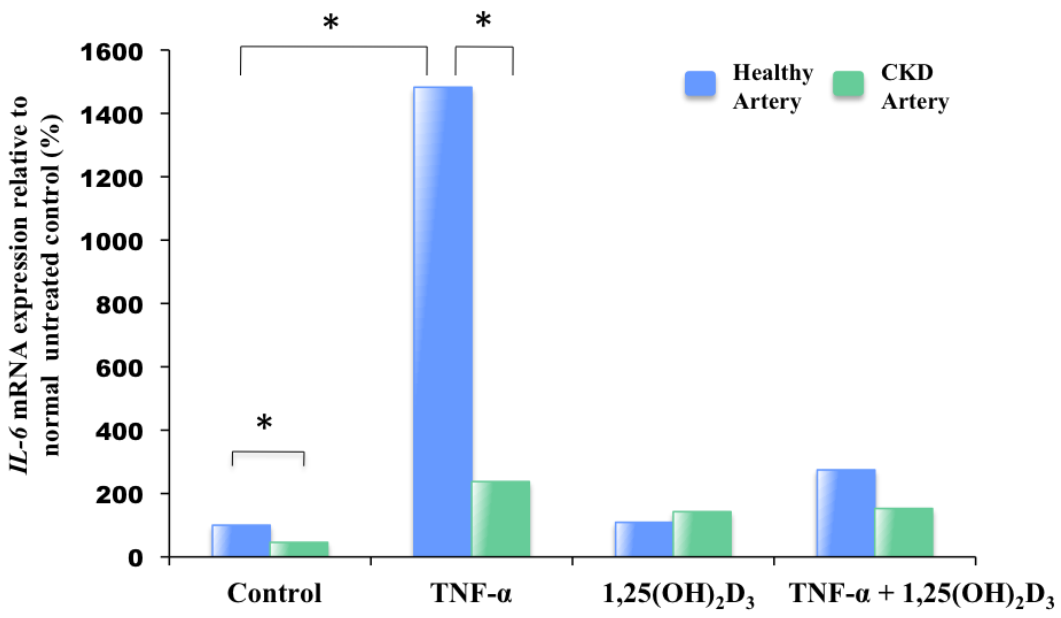


***Figure 5.11: The effect of TNF-α, 1,25(OH)$_2$D$_3$ and a combination of both on the IL-6 mRNA expression in healthy and Chronic Kidney Disease (CKD) arteries.*** *Human arterial explants were incubated with vehicle (0.1% ethanol) or TNF-α (20 ng/ml) or 1,25(OH)$_2$D$_3$ (100 nM) or the combination of both, for 48 hours. Differences in mRNA expression were examined by real-time RT-PCR analyses (normalised to 18S rRNA levels). Results represent mean of relative mRNA levels; n =11 healthy, 16 CKD, * p < 0.05; vs. healthy untreated control or vs. healthy treated control, as determined by one-way ANOVA. Data provided by Dr Guerman Molostvov.*

To determine the effect of 1,25(OH)$_2$D$_3$ on osteoblastic marker *RUNX-2* mRNA expression in presence of TNF-α and/or 1,25(OH)$_2$D$_3$ in arteries from healthy and CKD patients was assessed. Human arterial explants were incubated with vehicle (PBS with 0.1% BSA) or TNF-α (20 ng/ml) or 1,25(OH)$_2$D$_3$ (100 nM) or the combination of both, for 48 hours. *RUNX-2* mRNA levels were 175% higher in CKD arteries, compared to basal expression in untreated healthy controls ($p<0.01$). Treatment with TNF-α hugely induced (689%) *RUNX-2* mRNA in healthy arteries ($p<0.01$), but not in CKD. 1,25(OH)$_2$D$_3$ in presence of TNF-α had no effect on *RUNX-2* mRNA, 1,25(OH)$_2$D$_3$ on its own significantly induced *RUNX-2* mRNA

expression by 84% in normal arteries *(p<0.01)*, and decreased *RUNX-2* mRNA by 111% in CKD *(p<0.05) (Figure 5.12).*

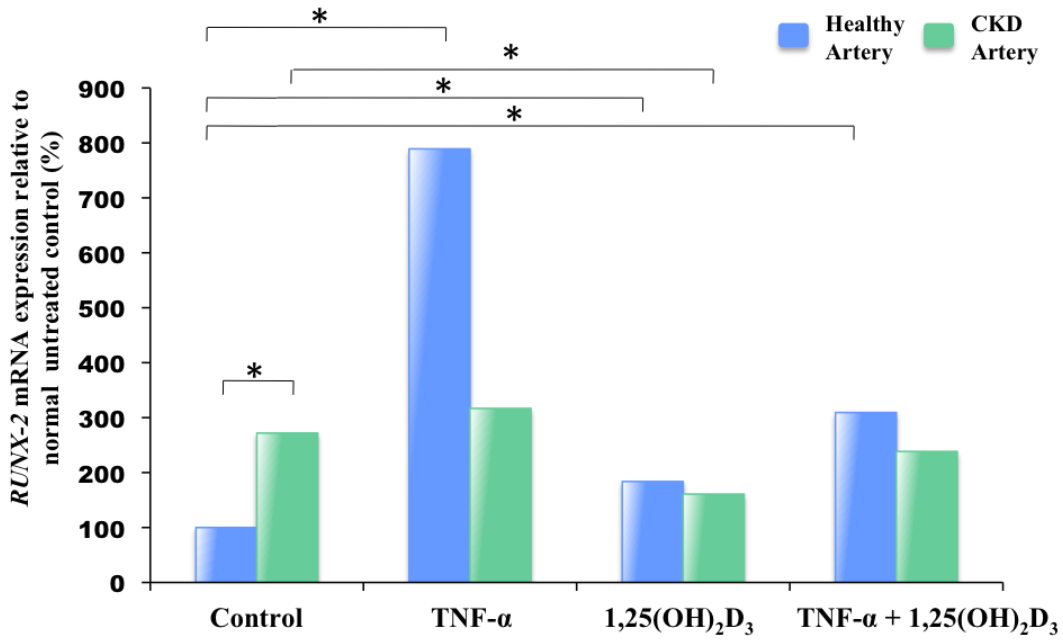


***Figure 5.12**: The effect of TNF-α, 1,25(OH)$_2$D$_3$ and a combination of both on the RUNX-2 mRNA expression in healthy and Chronic Kidney Disease (CKD) arteries. Human arterial explants were incubated with vehicle (0.1% ethanol) or TNF-α (20 ng/ml) or 1,25(OH)$_2$D$_3$ (100 nM) or the combination of both, for 48 hours. Differences in mRNA expression were examined by real-time RT-PCR analyses (normalised to 18S rRNA levels). Results represent mean of relative mRNA levels; n =11 healthy, 16 CKD, * p < 0.05; vs. normal untreated control or vs. normal treated control, as determined by one-way ANOVA. Data provided by Dr Guerman Molostvov.*

### 5.2.4 The Effect of INF-γ, IL-6 and IL-17A on *VDR*, *CYP27B1* and *CYP24A1* mRNA and Protein Expression in HAoSMCs

HAoSMCs were treated with INF-γ, IL-6 or IL-17A at concentrations, which have previously shown effect in SMC, *in vitro* (Agrawal *et al.* 2012, Eid *et al.* 2009, Geng *et al.* 1996, Pietrowski *et al.* 2011).

#### *5.2.4.1 INF-γ, IL-6 and IL-17A Suppressed CYP27B1 mRNA*

Further, studies using HAoSMCs demonstrated that INF-γ (20 ng/ml) or IL-6 or IL-17A (200 ng/ml) had no significant effect on *VDR* mRNA expression at 6 or 24 hours *(Figure 5.13A).* In contrast, the expression of *CYP27B1* mRNA was decreased by approximately 50% by exposure to INF-γ (*p= 0.01)* or IL-6 (*p= 0.04)* or IL-17A (*p= 0.049*), at 24 hours. There were no significant changes in *CYP27B1* mRNA expression at 6 hours *(Figure 5.13B).* INF-γ or IL-6 or IL-17A had no effect on *CYP24A1* mRNA expression *(Figure 5.13C).*

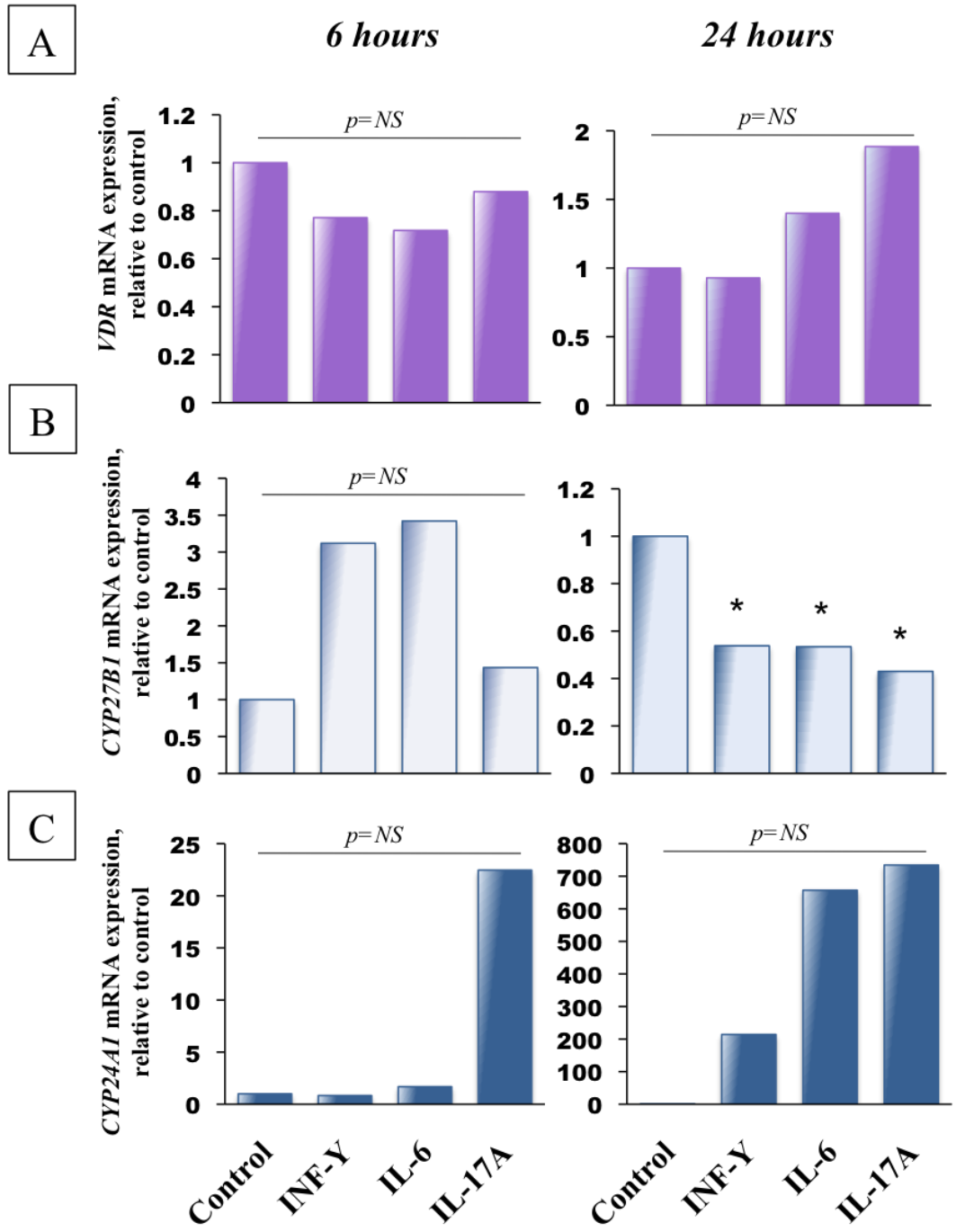


***Figure 5.13: The effect of INF-γ, IL-6 and IL-17A on the Vitamin D Receptor (VDR), 1α-Hydroxylase (CYP27B1) and 24-Hydroxylase (CYP24A1) mRNA expression in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *Cells were treated with vehicle (PBS with 0.1% BSA), INF-γ (20 ng/ml), IL-6 (200 ng/ml) and IL-17A (200 ng/ml) for 6 and 24 hours. (A) VDR mRNA expression (B) CYP27B1 mRNA expression (C) CYP24A1 mRNA expression, as demonstrated by real-time RT-PCR analyses. Readings were corrected to 18S rRNA expression levels. Results represent mean of relative mRNA levels, n =4, * $p < 0.05$; vs. control as determined by one-way ANOVA.*

#### *5.2.4.2 INF-γ Modulated 1α-OHase and 24-OHase Protein Expression*

Primary cultures of HAoSMc were incubated with vehicle (PBS with 0.1% BSA) or high concentrations of individual cytokines (INF-γ: 20 ng/ml; IL-6: 200 ng/ml; IL-17A: 200 ng/ml) and changes in protein expression of 1α-OHase, 24-OHase and the VDR, at 6 hours and at 24 hours were examined by Western blot analyses. Neither VDR *(Figure 5.14A)*, 1α-OHase *(Figure 5.14B)* nor 24-OHase protein expression *(Figure 5.14C)* was affected by any of the cytokines at 6 hours. However, following 24 hours of treatment 1α-OHase protein expression was significantly upregulated by INF-γ 1.6-fold (*p= 0.006*) *(Figure 5.14B).* 24-OHase protein expression was significantly decreased by INF-γ, 0.4-fold (*p= 0.007*) at 24 hours *(Figure 5.14C).* No other significant changes in 1α-OHase, 24-OHase or VDR protein expression were induced *(Figure 5.14).*

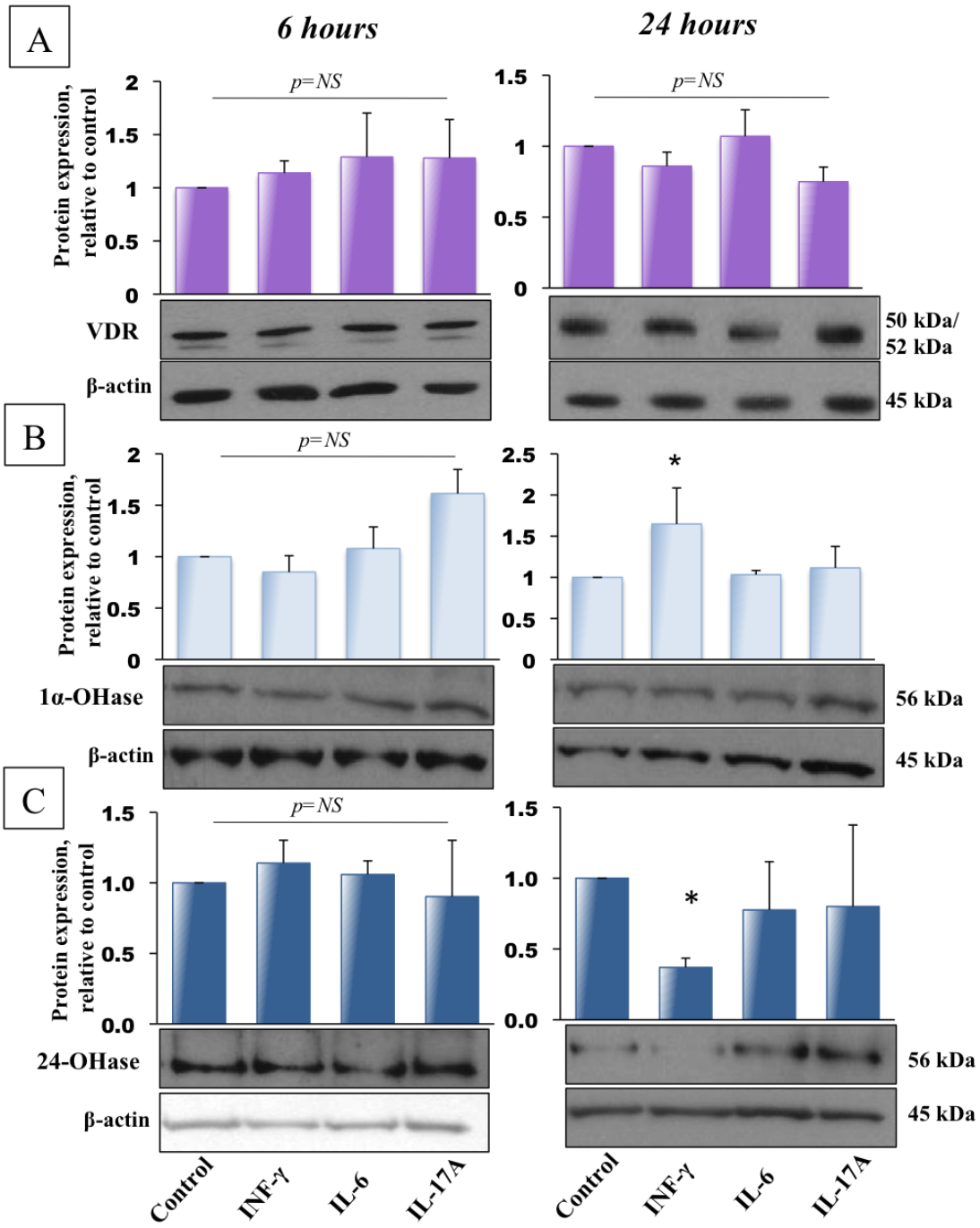


***Figure 5.14: The effect of INF-γ, IL-6 and IL-17A on the Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *Cells were treated with vehicle (PBS with 0.1% BSA), INF-γ (20 ng/ml), IL-6 (200 ng/ml) and IL-17A (200 ng/ml) for 6 and 24 hours. Results represent mean±SEM, n =3, *p <0.05, as determined by one-way ANOVA; each graph is accompanied by representative Western blots (A) VDR (B) 1α-OHase (C) 24-OHase. β-actin was used for equal loading.*

## 5.3 Discussion

Results presented in this chapter clearly demonstrated three major findings. Firstly, increased calcium upregulates VDR protein expression in VSMC, as well as 1α-OHase protein and 24-OHase. Secondly, although phosphate has no effect on VDR protein expression, it stimulates 1α-OHase and 24-OHase expression. Lastly, TNF-α induces both VDR and 24-OHase protein expression in VSMCs. In healthy human artery TNF-α increases mRNA expression of *VDR*, CYP27B1 and *CYP24A1*. $1,25(OH)_2D_3$ blunts the effect TNF-α has on the expression of vascular vitamin D system in healthy artery, but not in CKD artery - action advantageous to limit inflammation.

The main conclusion from all the above findings is that the role of VDR as a target for $1,25(OH)_2D$ in VSMC is crucial – it is important for local $1,25(OH)_2D$ production, for feedback regulation through acting on 1α-OHase and 24-OHase and also for the endocrine $1,25(OH)_2D$ and importantly, the pharmacological therapy with active vitamin D compounds. CKD is characterized by an altered calcium and phosphate metabolism on a background on inflammation, and these clearly have effects on VSMCs, and although vitamin D signalling is intact in CKD it is altered and probably attenuated. The impact of stressors such as calcium, phosphate or TNF-α on VSMCs VDR's function can have deleterious consequences. Changes in cell can occur as a result of stressor induced - altered gene expression (Wu-Wong *et al.* 2007b, c), eventually contributing to phenotypic changes (Jono *et al.* 2000, Reynolds *et al.* 2004, Tintut *et al.* 2000). I have shown that 2 and 3 mM calcium increased 1α-OHase protein expression as early as 6 hours and the increase was sustained by at least 48 hours. One explanation could be transformation of cells under calcium

stress. However my earlier findings from studies in CKD artery where decreased levels of 1α-OHase were observed would contradict this theory, it is important to note that the experimental models are different – HAoSMCs treated with calcium represent more of an acute model, as the cells were obtained from healthy donors and were not previously exposed to long-term stressors to simulate CKD. The differentiation stage of cells can also play a role. Interestingly calcium had similar effect on both 1α-OHase and 24-OHase protein expression. Perhaps such feature is typical of autocrine regulation. From observations in other models such as keratinocytes, placenta and macrophages it could be hypothesised that local synthesis of $1,25(OH)_2D_3$ is controlled not only by suppression of 1α-OHase but also by the supplementary action of upregulated 24-OHase (Bacchetta J. *et al.* 2013, Novakovic *et al.* 2009, White 2012, Xie *et al.* 2002). Upregulation of locally expressed 1α-OHase by calcium has been observed in other extra-renal sites - in bone and in parathyroid. In kidney proximal tubule cells, 4 –hour treatment with 0.5 mM calcium resulted in 4.8-fold increase in *CYP27B1* mRNA. 2 mM on the other hand, significantly decreased *CYP27B1* mRNA expression and activity at 6 and 10 hours. Similar pattern was described in *CYP24A1* mRNA expression (Bland *et al.* 1999). Studies using hyperplastic ureamic parthyroid tissue demonstrated that exposure to 2.7 mM calcium for 24 hours induced a 50% increase in *CYP27B1* mRNA expression and over 100% increase in *CYP24A1* mRNA expression (Ritter *et al.* 2012). Calcium has previously been shown to induce human macrophage *CYP27B1* mRNA *in vitro*, and to inhibit the renal enzyme (Hewison *et al.* 2000). However, the fact that high calcium has directly opposite effect on renal *CYP27B1* mRNA expression and activity, compared to parathyroid and bone, may suggest that calcium or phosphate act more like immune stimuli in extra-renal setting.

Phosphate did not induce changes in VDR expression, however it significantly induced protein levels of 1α-OHase and 24-OHase. Perhaps different effect on VDR was to be observed had the concentration of phosphate in the treatment medium was higher or time of treatment was extended. The concentration of calcium in the treatment medium may also play a role. Previous studies looking at calcium incorporation in VSMCs demonstrated that only 4 or 5 mM phosphate had significant effect at 24 hours, however when concentration of both was increased to 2.6 mM (Ca) and 2 mM (P), this was also sufficient to significantly induce calcium incorporation (Reynolds *et al.* 2004). When looking at the effect phosphate exerted on 1α-OHase and 24-OHase protein expression, it becomes apparent that there is a clear approximately 24-hour delay in the induction of 24-OHase. This could be attributed to combating of the initial raise in local $1,25(OH)_2D$ levels.

Results presented in this chapter confirmed that TNF-α at 20 ng/ml induced VDR protein in HAoSMCs, but the observed increase in *VDR* mRNA was not statistically significant. Similarly, *CYP27B1* and *CYP24A1* mRNA and protein did not follow the same pattern, suggesting possible involvement of micro RNAs. TNF-α induced *VDR* mRNA by 10-fold in healthy arteries, compared to vehicle treated controls. These results directly contradict the recently published studies in which Agrawal and colleagues demonstrated the opposite effect of modulation of human bronchial SMCs with TNF-α, at a concentration of just 10 ng/ml, which is lower than I used (Agrawal *et al.* 2012). Differential regulation in the same cells but in different organs in the body may be a possible explanation. Perhaps SMCs of human bronchi are more sensitive to cytokine changes, in order to more robustly control allergic inflammation of the airway. On the other hand, VDR activation observed in both HAoSMCs and normal arteries may serve as a protective mechanism, through induction of

$1,25(OH)_2D_3$ synthesis and repression of the proinflammatory INF-γ gene transcription.

Evidence as to whether calcium or phosphate is more potent stimulus in induction of medial calcification is contradictory. Some suggests that elevated calcium is more potent than elevated phosphate in human arteries, *ex vivo* (Shroff R. C. *et al.* 2010). Others present evidence which favors phosphate (Jono *et al.* 2000, Kendrick and Chonchol 2011, Wu-Wong *et al.* 2006c). In CKD contractile VSMCs, in order to cope with calcium overload, progressively differentiate into vesicle secreting cells. The presence of functional calcification inhibitors in vesicles ensures protection from calcification (Reynolds *et al.* 2004, Shanahan 2006, Shroff R. C. *et al.* 2008). Exposure of such differentiated cells to high calcium for prolonged period of time leads to calcium-induced apoptosis (Shanahan 2006). Since $1,25(OH)_2D_3$ has been previously shown to have an anti-apoptotic activity in murine osteoblasts and osteocytes (Vertino *et al.* 2005), it is also possible that a local increase in 1α-OHase in response to high calcium or phosphate concentrations is a part of an anti-apoptotic protective cellular response.

Previous studies performed on partially nephrectomised ApoE-deficient mice have demonstrated that the uremic milieu per se may accelerate atherogenesis (Buzello *et al.* 2003). Results presented in this chapter confirmed that in arteries from CKD patients *VDR* mRNA expression was not induced by TNF-α. This may be attributed to complex phenotypic changes in CKD VSMCs. Interesting observations were made in our studies on the expression of *CYP27B1* mRNA and *CYP24A1* in CKD arteries treated with TNF-α. *CYP27B1* mRNA expression in normal arteries was increased in presence of TNF-α by 2-fold, and in CKD arteries the same concentration of TNF-α induced a 6-fold increase in the transcript levels of this enzyme. An increase in 1α-

OHase activity post-TNF-α challenge has previously been observed in human umbilical vein endothelial cells (Zehnder *et al.* 2002b). A directly opposite effect to that for *CYP27B1* mRNA was recorded for *CYP24A1* in CKD vessels, ultimately suggesting that the hampered VDR signalling in CKD may have a knock-on effect on uncontrolled increase in *CYP27B1* mRNA.

In conclusion, suppression of the renal 1α-OHase by high levels of calcium is part of a feedback loop to reduce $1,25(OH)_2D_3$-mediated intestinal calcium absorption. A similar suppression of the 1α-OHase in the VSMCs and parathyroid glands by high calcium would diminish endogenous $1,25(OH)_2D_3$ production and could be counterproductive to calcium feedback on circulating PTH, but also could be counterproductive to regulation of proliferation, differentiation and apoptosis. Results presented in this chapter may also suggest that induction of the 1α-OHase may be an additional, indirect fine-tuning mechanism for the control of PTH by calcium and calcium receptor activators.

In the light of the research presented in this thesis, it is becoming apparent that VSMCs resemble the immune cell model by regulating their $1,25(OH)_2D_3$ synthesis through responses to mitogens including TNF-α. Thus, the data presented suggest that the presence of the intact functional local vitamin D system in human VSMCs may be a prerequisite for maintenance of cellular protection through synergistic actions with cytokines. The results propose that the mineral and inflammatory disruption of systemic vitamin D homeostasis have a knock on effect on the local vitamin D metabolism, a disruption of which can in consequence lead to inappropriate control/responses to chronic/prolonged changes in the local cytokine levels.

# Chapter 6

# Novel Regulators of the Vitamin D System in Human Artery and VSMCs: FGF-23 and Klotho

## 6.1 Background

Deterioration of renal function in CKD is accompanied by progressively increasing levels of serum FGF-23, a mechanism evolved to possibly regulate high phosphate. In humans, loss of FGF-23 leads to hyperphosphatemia, increased serum $1,25(OH)_2D_3$, as well as ectopic soft tissue calcifications (Benet-Pages *et al.* 2005, Topaz *et al.* 2004). Klotho knockout mice ($Kl^{-/-}$) have multiorgan premature ageing, hyperphosphatemia, hypercalcemia and associated medial calcification, together with elevated $1,25(OH)_2D_3$ and FGF-23 (Brownstein *et al.* 2008, Ichikawa *et al.* 2007, Kuro-o *et al.* 1997, Segawa *et al.* 2007). Treatment of mice with a cleaved variant of Klotho results in hypophosphataemia and hypocalcaemia combined with huge upregulation of FGF-23 (Smith R. C. *et al.* 2012). For its action in any target tissue, FGF-23 requires Klotho in order to bind and activate the cognate FGF receptors with sufficient affinity (Yu *et al.* 2005). Interestingly, recent evidence demonstrated that Klotho does not require action of FGF-23 to induce phosphaturia, as it exhibits a direct phosphaturic effect itself (Hu *et al.* 2010). In CKD patients on haemodialysis,

possession of a genetic variant of the *Klotho* gene (rs577912) has been associated with survival (Friedman *et al.* 2009). FGF-23 levels start increasing early in CKD and even the high end of the normal range has been associated with arterial stiffness and impaired vasoreactivity (no decrease in pulmonary artery pressure in response to vasodilator challenge) in humans (Mirza *et al.* 2009a, Mirza *et al.* 2009b). Apart from inhibiting 1α-OHase and so $1,25(OH)_2D$ synthesis in the kidney, increased levels of FGF-23 have been associated with the reduction of Klotho expression (Krajisnik *et al.* 2007, Shimada *et al.* 2004b).

FGF-23 also acts on tissues other than the kidney, which synthesise $1,25(OH)_2D_3$, such as monocytes. Studies on two different human monocyte models (healthy donor peripheral blood mononuclear cell monocytes (PBMCm) and peritoneal dialysate monocyte (PDm) effluent from kidney disease patients) revealed that FGF-23 suppressed *CYP27B1* reducing hydroxylation of 25(OH)D to $1,25(OH)_2D$, which is accompanied by suppression of *CYP24A1* mRNA expression and antibacterial cathelicidin (Bacchetta J. *et al.* 2013). These findings suggest that in CKD local responses to $1,25(OH)_2D$ can be inhibited by the action of FGF-23 on local monocyte vitamin D metabolism.

Since VSMCs express Klotho and FGFRs, they are potentially a target tissue for FGF-23. Membrane bound (mb) and/or soluble (s) Klotho act as co-factors for FGF-23, by converting FGFR-1 into a specific receptor for FGF-23. This allows binding of the FGF-23 to Klotho and the FGFR-1. The Klotho-FGF interaction is thought to occur at the FGF-23 C-terminal (Goetz *et al.* 2007). This interaction results in induction of phosphorylation cascade and activation of extracellular signal-regulated protein kinase (ERK1/2). ERK1/2 could further influence the regulation of early growth response protein 1 (Egr-1) and Nab-2 (Egr-1 binding protein), which allows a

negative feedback loop. Egr-1 family of proteins are zinc-finger nuclear proteins, which activate promoters of genes responsible for VSMC proliferation and responses to sheer stress or vascular injury. Co-repressors, such as Nab-1 and Nab-2 negatively regulate the Egr-1 activity, possibly maintaining the VSMC homeostasis (Silverman *et al.* 1999). Signalling via ERK1/2 could also result in a downstream phosphorylation of VDR and induction of transcription from VDREs. It is unclear whether endogenously expressed Klotho can exert actions similar to FGF-23, *in vivo*. In the light of recent research, it is not unreasonable to assume that Klotho may modulate transcription from VDRE via second messengers such as cAMP or via direct interactions with VDR. Most recent findings suggest that FGF-23-Klotho axis in vasculature plays an important role in maintaining vascular health (Donate-Correa *et al.* 2011, Hu *et al.* 2011, Lim *et al.* 2012). There are conflicting data on whether FGF-23 has vasculoprotective effects in CKD. The recent findings of Shalhoub and co-workers indicated that the neutralization of FGF-23 in CKD rat model, despite improving CKD-associated hyperparathyroidism, increased mortality (Shalhoub *et al.* 2012). Further, most recent data from studies on chronic renal insufficiency cohort of 1501 patients and *in vitro* studies on VSMCs support the notion that in contrast to serum phosphate, FGF-23 is not associated with arterial nor VSMC calcification, suggesting that FGF-23 may contribute to cardiovascular complications via alternative mechanisms (Scialla *et al.* 2013). Studies in mice with partial Klotho knockout limited to and specific to distal tubule suggested that high FGF-23 detected in these mice implied that human CKD is a state of FGF-23 resistance (Olauson *et al.* 2012). However there are strong arguments against partial Klotho deletion being an accurate representation of CKD due to the inconsistencies between the experimental

mouse and the human CKD phenotype, including $1,25(OH)_2D$ levels (Isakova and Wolf 2012).

To date the collective evidence suggests that FGF-23 is not only involved in the bone-kidney axis, but also the bone-parathyroid and intestine-kidney axis (Ben-Dov *et al.* 2007, Razzaque and Lanske 2007). In this chapter the possible bone-vasculature axis was investigated from the perspective of local vitamin D metabolism in VSMCs.

In chapter 5 I demonstrated that high phosphate (2mM) induced increase in endogenous vascular 1α-OHase protein expression by 24 hours and induced an even more significant increase in 24-OHase protein in HAoSMCs. Further I showed that 1α-OHase protein levels returned to basal levels after 48 hours. These findings suggested that this might be attributed to the action of an endogenous FGF-23 and/or Klotho potentially synthesised by VSMCs. High phosphate initially induced an increase in 1α-OHase as a vasculoprotective mechanism, this may have also induced FGF-23 and/or Klotho, which acted to decrease phosphate load, and as the mineral balance has been reestablished, 1α-OHase protein levels dropped back to basal level. The observable increase in 24-OHase protein expression is an expected negative feedback regulation of 1α-OHase synthesis.

The above hypothesis has prompted me to investigate whether the observed changes were in fact attributed to the endogenous action of FGF-23 and/or Klotho. Therefore, in this chapter the effect of these novel vitamin D system regulators on the expression of VDR, 1α-OHase and 24-OHase protein in HAoSMCs were examined to better understand their possible contribution in the maintenance of vascular health.

## 6.2 Results

### 6.2.1 Regulation of VDR, 1α-OHase and 24-OHase Protein Expression by FGF-23 in HAoSMCs

#### *6.2.1.1 Identification of the FGF Receptor 1 and FGF Receptor 3 protein in HAoSMCs*

Western blot analyses confirmed the presence of both FGF receptor 1 (FGFR1) and 3 (FGFR3) protein in primary cultures of HAoSMCs (*Figure 6.1).* FGFR3 appeared to be more abundant than FGFR1. It was not possible to investigate the protein expression of FGFR2, as the antibody used did not produce reliable results. The presence of mRNA for FGF receptors: *FGFR1*, *FGFR2* and *FGFR3* has been confirmed recently in human aorta (Donate-Correa *et al.* 2011) and FGFR1 and FGFR3 protein in primary cultures of VSMCs (Lim *et al.* 2012).

#### *6.2.1.2 Klotho Protein Variants are Expressed in HAoSMCs*

Cells were grown in basal medium without FCS or with 0.5% FCS, or without FCS, but in presence of exogenous human recombinant hrKlotho (60-70 kDa) (200 pM, 24 hours). Western blot analyses confirmed that HAoSMCs express membrane bound - mbKlotho (130 kDa), secreted - sKlotho (80 kDa) and cleaved - cKlotho (68 kDa) proteins. In the absence of FCS, HAoSMCs expressed only mbKlotho (130 kDa). Expression of sKlotho and cKlotho was stronger in cells grown with FCS and weaker in cells treated with hrKlotho (*Figure 6.2*)

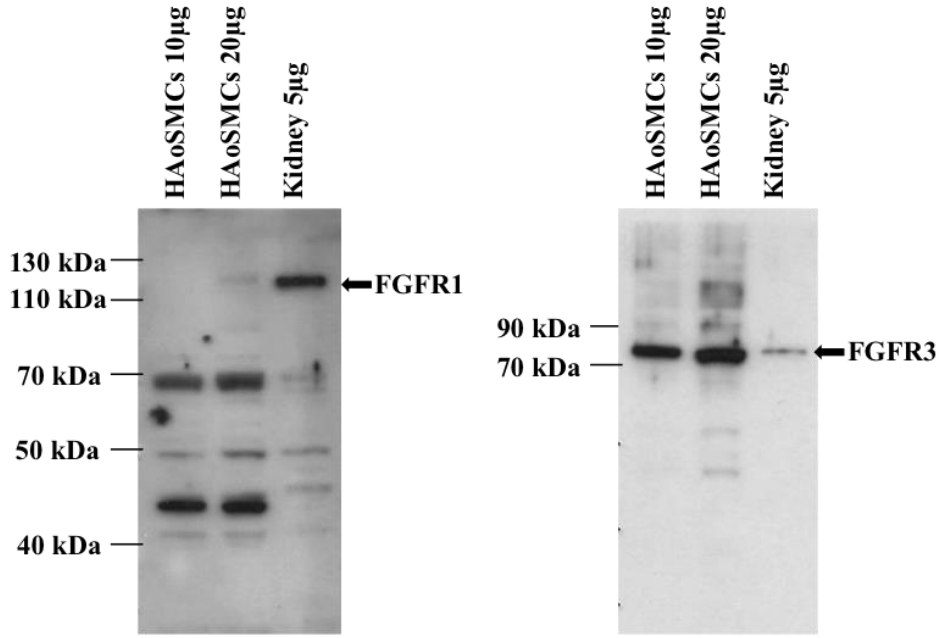


***Figure 6.1: The Fibroblast Growth Factor (FGF) Receptor 1 (FGFR1) and FGF Receptor 3 (FGFR3) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *FGFR1: a weak band of approximately 115 kDa corresponding to the one seen in kidney was detected in HAoSMCs, as demonstrated by Western blot analysis. FGFR3: a strong band of about 80 kDa was detected in HAoSMCs. Human kidney was used as a positive control.*

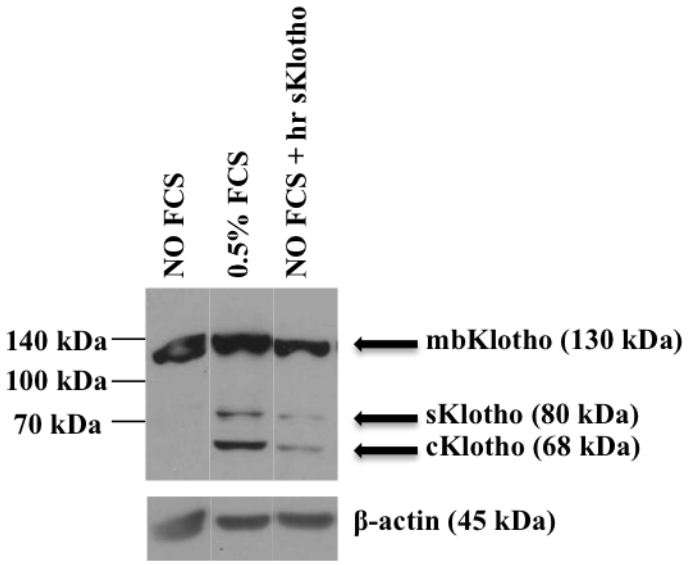


***Figure 6.2: Expression of Klotho protein in Human Aortic Smooth Muscle Cells (HAoSMCs).*** *Cells were grown in basal medium with no foetal calf serum (FCS) or with 0.5% FCS, or without FCS, but in presence of exogenous human recombinant (hr) soluble sKlotho (60-70 kDa) (200 pM, 24h). mb- membrane bound, s- secreted, c- cleaved Klotho protein. It appears that in absence of FCS, HAoSMCs express only mbKlotho (130 kDa). s- and cKlotho (80 kDa and 68 kDa, respectively) appear to be present in cells grown with FCS and to a lesser extent in cells treated with hr sKlotho.*

### *6.2.1.3 The FGF-23 Signalling in HAoSMCs is via ERK1,2 Phosphorylation Pathway*

Further investigation demonstrated that the treatment of HAoSMCs with 100 ng FGF-23 induced phosphorylated ERK (Phospho-ERK1,2) protein expression (42 and 44 kDa), reaching a peak at 30 minutes compared with the vehicle (0.1% DMSO) treated control, as demonstrated by Western blot analysis (*Figure 6.3*). Inhibition was achieved by pre-incubating the cultures with 60 μM of a MEK-1 inhibitor (PD98059) for 1 hour prior to treatment with FGF-23 for 30 minutes. Phorbol 12-myristate 13-acetate (PMA) at 50 μM for 5 minutes was used as a positive control of ERK phosphorylation. Treatment of cells with PMA in the absence of FGF-23 and PD98069 resulted in a strong upregulation of phosphorylated ERK-1,2, compared to vehicle treated control.

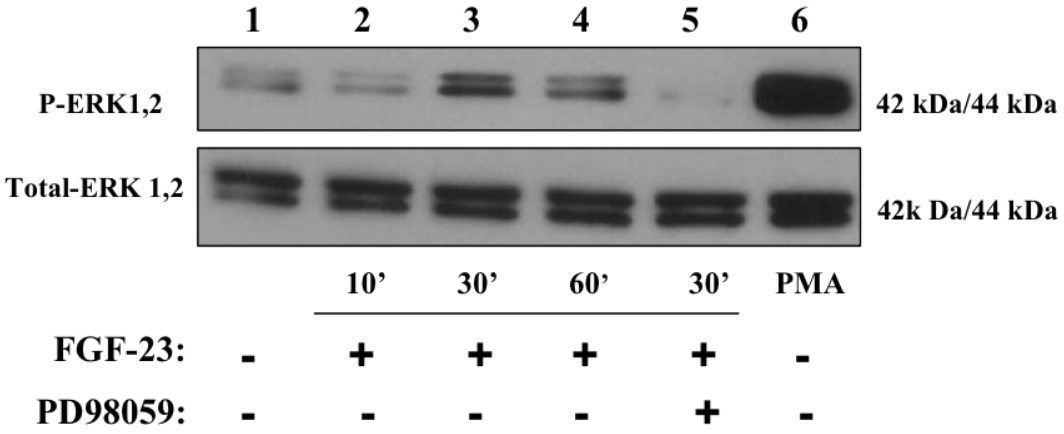


***Figure 6.3: The effect of Fibroblast Growth Factor-23 (FGF-23) on signal transduction via the MAPK Pathway.*** *HAoSMCs were treated with vehicle (0.1% BSA) or FGF-23 (100ng/ml) for 10, 30 and 60 minutes. For lane 5, cells were incubated with MEK-1 inhibitor (PD98059) (60 μM) 30 minutes before exposure to FGF-23, for lane 6 cells were incubated with phorbol 12-myristate 13-acetate (PMA) as a positive control for ERK phosphorylation. Cell lysates were prepared in the presence of phosphatase inhibitors, and phosphorylated proteins were detected by chemiluminescence using rabbit anti-phospho-ERK1,2 antibodies. Equal protein loading was monitored by detection of total-ERK1,2 protein.*

#### *6.2.1.4 FGF-23 Induces VDR and 24-OHase Protein Expression, in the Absence of FCS*

Previous studies conducted by our group and others have demonstrated the presence of endogenous *Klotho* mRNA and protein in vasculature (Donate-Correa *et al.* 2011, Lim *et al.* 2012). To test whether Klotho is essential for the action of exogenous FGF-23, cells were treated in absence of serum (to avoid Klotho which may be present in the serum). HAoSMCs were treated with vehicle (0.1% BSA) or 5 ng/ml FGF-23 (concentration previously optimized by Perwad in HKC-8 and mouse renal proximal tubule cells (MCT) (Perwad *et al.* 2007) and Lim in HAoSMCs (Lim *et al.* 2012), also ED50 suggested by the supplier). Serum concentration of FGF-23 in healthy adults is 0.004 ng/ml ± 0.00017 (Yamazaki *et al.* 2010), reaching about 50 ng/ml in early CKD (Larsson *et al.* 2003) and about or over 100 ng/ml in advanced stages of CKD (Dai *et al.* 2012). Treatment medium was changed every 24 hours.

Western blot analyses demonstrated that FGF-23 induced about a 50% increase in VDR protein expression across all time points, but only the 24-hour treatment resulted in a significant change (*Figure 6.4*).

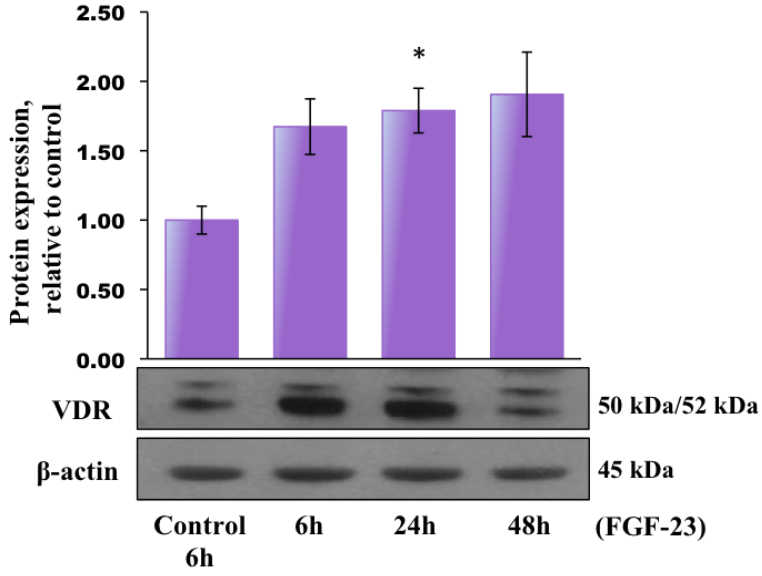


***Figure 6.4: The effect of the Fibroblast Growth Factor (FGF) 23 on the Vitamin D Receptor (VDR) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *Cells were treated with vehicle (0.1% BSA) or 5 ng/ml FGF-23 for 6, 24 and 48 hours, as demonstrated by Western blot analyses. Results are presented as ratio of VDR protein expression and corresponding β-actin protein levels. Results represent mean ± SEM, n = 6, * p < 0.05; vs. control; as analysed by one-way ANOVA.*

No significant changes in 1α-OHase protein expression were observed following FGF-23 treatment of HAoSMCs for 6, 24 or 48 hours *(Figure 6.5A)*. In contrast, 24-OHase protein expression, was significantly induced by 3-fold following 48 hours of treatment *(Figure 6.5B)*.

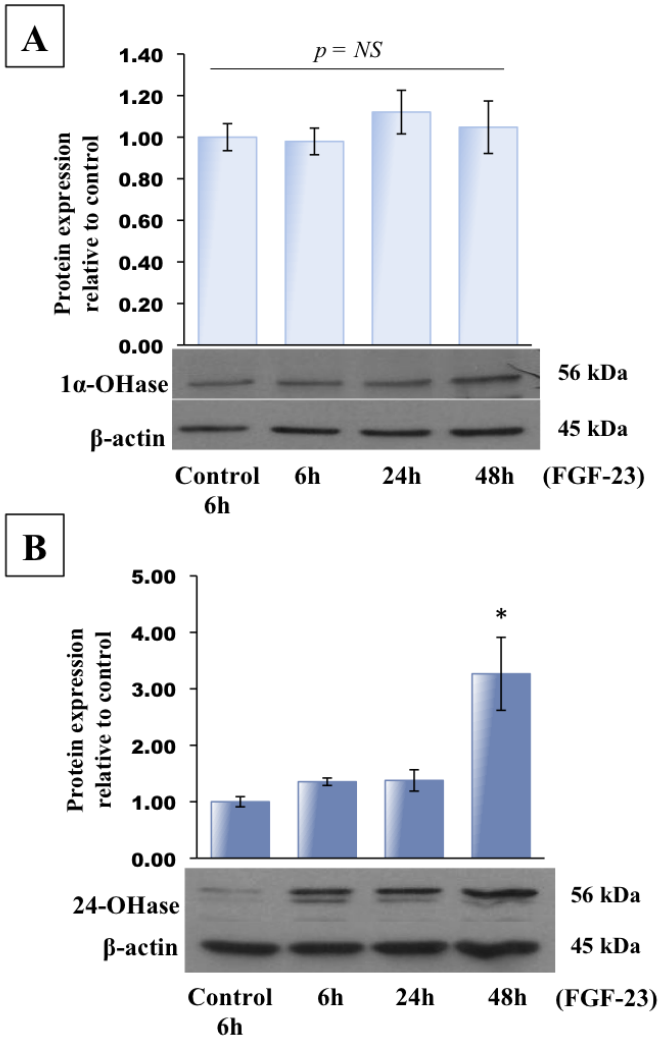


***Figure 6.5: The effect of the Fibroblast Growth Factor-23 (FGF-23) on the 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) in absence of Foetal Calf Serum (FCS) over time***. *Cells were treated with vehicle (0.1% BSA) or 5 ng/ml FGF-23 for 6, 24 or 48 hours. (A) 1α-OHasee protein expression (B) 24-OHase protein expression, as demonstrated by Western analyses. Results are presented as ratio of 1α-OHase or 24-OHase protein expression and corresponding β-actin. Results represent mean ± SEM, n = 6, * p < 0.05; vs. control; one-way ANOVA. Graphs are presented with representative Western blots.*

### *6.2.1.5 FGF-23 Suppresses 1α-OHase in the Presence of FCS*

To test whether the previously observed lack of change in 1α-OHase protein expression following treatment with FGF-23 was real, or whether the presence of FCS, which might contain Klotho and 25(OH)D alters responses, an alternative set-up was examined. Cells were treated with vehicle (0.1% BSA) or normal (5 ng/ml)

or high (100 ng/ml) dose of FGF-23 in absence or in presence of 0.5% FCS, for 24 and 48 hours. FGF-23 doses of 5 ng/ml and 100 ng/ml have been used previously by others (Bacchetta J. *et al.* 2013, Ben-Dov *et al.* 2007, Lim *et al.* 2012, Perwad *et al.* 2007). The treatment of HAoSMCs with 100 ng/ml FGF-23 (48 hours) significantly inhibited 1α-OHase protein expression, however only in presence of FCS *(Figure 6.6).*

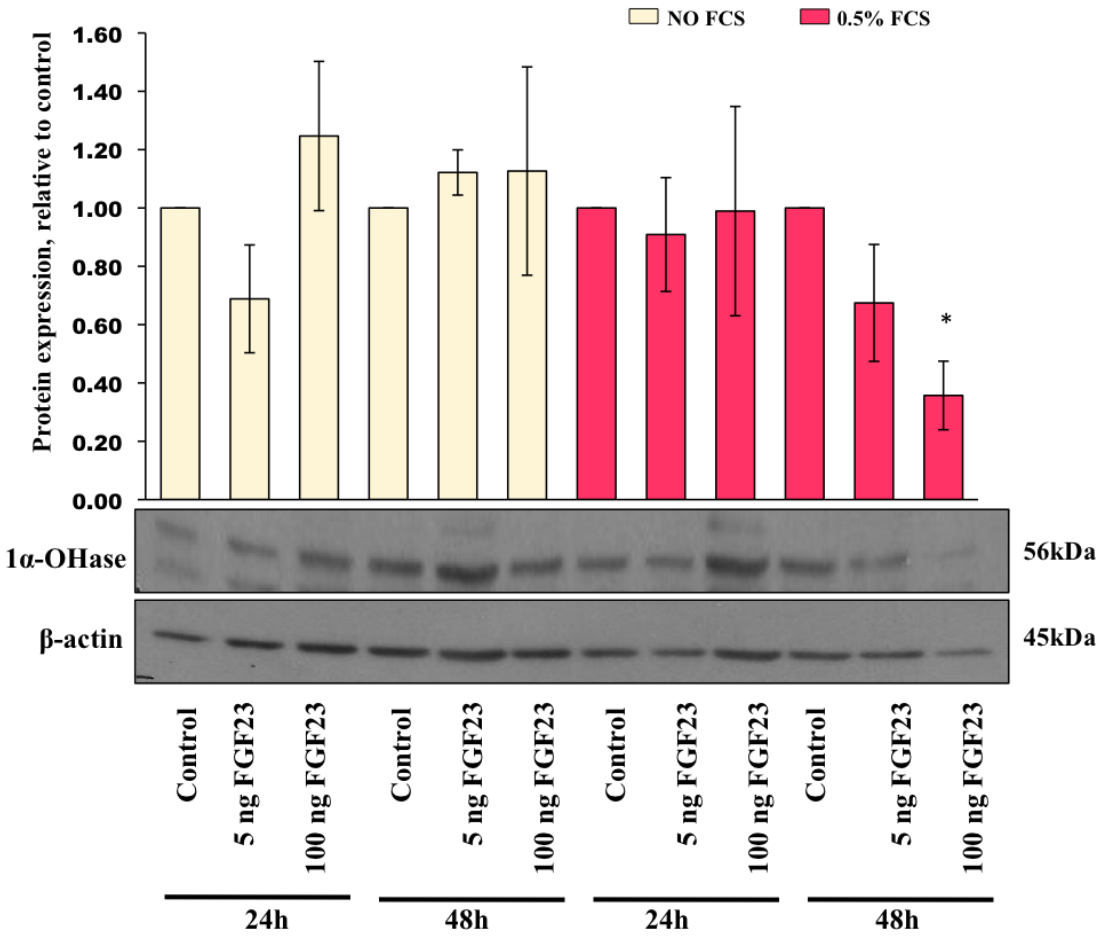


***Figure 6.6: The effect of the Fibroblast Growth Factor-23 (FGF-23) on the 1α-Hydroxylase (1α-OHase) protein expression in absence or presence of Foetal Calf Serum (FCS) in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *Cells were treated with vehicle (0.1% BSA) or 5 ng/ml or 100 ng /ml FGF-23 in absence or presence of 0.5% FCS for 24 and 48 hours. Results are presented as ratio of 1α-OHase protein expression and corresponding β-actin protein levels, as demonstrated by Western blot analyses. Results represent mean ± SEM, n = 4, * p < 0.05; vs. control; data were analysed by one-way ANOVA. Below the graph are the representative Western blots.*

### 6.2.2 Regulation of the VDR, 1α-OHase and 24-OHase Protein Expression by hr sKlotho in HAoSMCs

Previous studies by Hu and co-workers (Hu *et al.* 2010) proposed a model in which Klotho can function in the apical membrane of the proximal tubule as a direct extracellular enzyme deglycosylating NaP1-2a protein and/or a putative regulatory protein to reduce co-transport activity. Evidence supporting the model was a result of experiments on FGF-23-null mice injected with intravenous recombinant extracellular domain Klotho (64 pM), which responded by lowering phosphate in the serum and increasing phosphate excretion in the urine (Hu *et al.* 2010).

In order to test whether exogenous hr sKlotho exerts a direct action on VDR, 1α-OHase or 24-OHase protein expression, HAoSMCs were treated with vehicle (0.1% BSA) or increasing concentrations of hr sKlotho (200 pM, 400 pM and 600 pM) (Liu H. *et al.* 2007) with heparan sodium (10 μg/ml) (Allen and Rapraeger 2003) for 6, 24 and 48 hours. Serum levels of sKlotho in healthy adults range between 100-200 pM (Yamazaki *et al.* 2010), however the local tissue concentrations may be higher.

The concentration of 200 pM was shown to be optimal (*Figure 6.7*).

Heparan salts have previously been shown to stabilize FGF-23/Klotho/Receptor formation (Allen and Rapraeger 2003), however it is only recently that data have emerged suggesting that their presence is dispensable and does not affect the complex formation (Goetz *et al.* 2012).

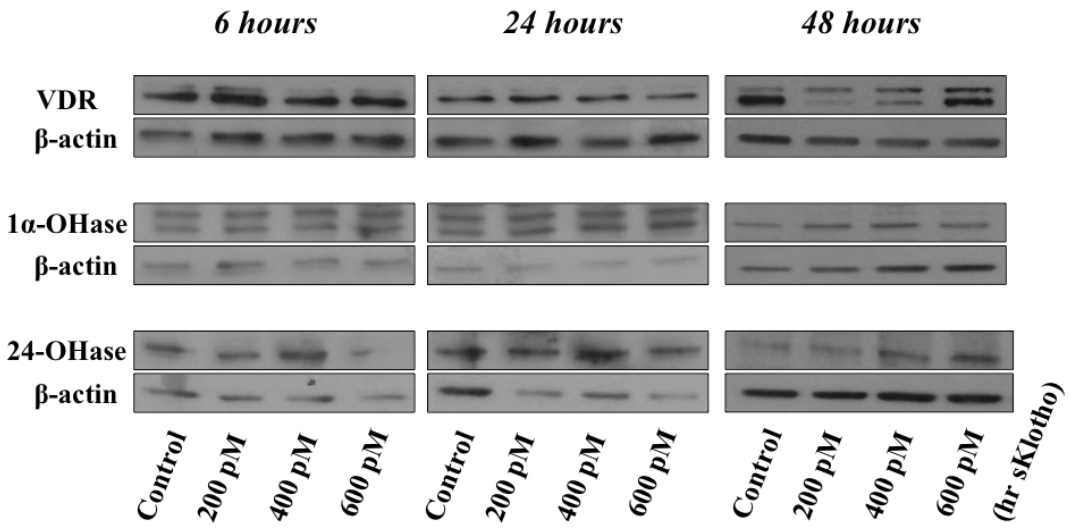


***Figure 6.7: The effect of increasing doses of the exogenous human recombinant soluble Klotho (hr sKlotho) on the Vitamin D Receptor (VDR), 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *Cells were treated with vehicle (0.1% BSA) or increasing concentrations of Klotho (200 pM, 400 pM and 600 pM) with heparan sodium (10 μg/ml) for 6, 24 and 48 hours. Concentration of 200 pM has been chosen as optimal for further time dependent studies.*

#### *6.2.2.1 sKlotho Mirrors FGF-23 by Independently Inducing the VDR and 24-OHase Protein Expression*

Western blot analyses demonstrated that hr sKlotho (200 pM) significantly up-regulated VDR protein expression (2-fold) at 6 hours, compared to vehicle treated control *(Figure 6.8).* *s*Klotho did not induce changes in 1α-OHase protein expression, 24-OHase protein on the other hand was significantly induced at 6 hours and remained slightly elevated at 24 and 48 hours *(Figure 6.9).*

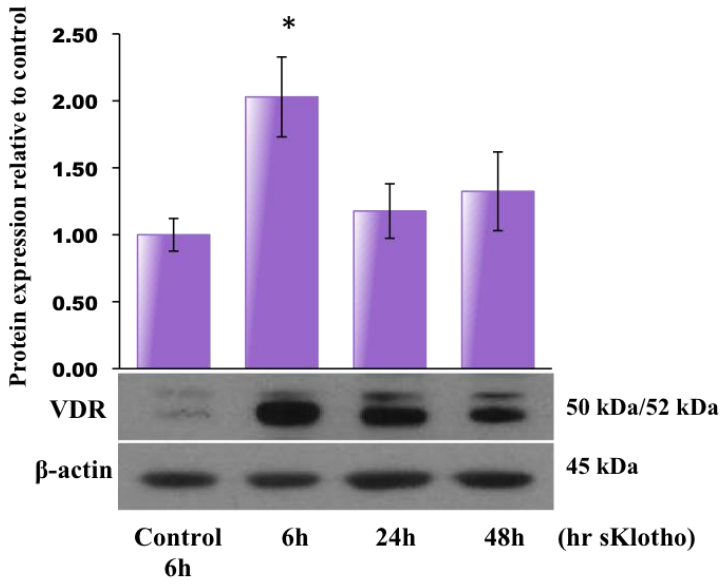


***Figure 6.8: The effect of human recombinant soluble Klotho (hr sKlotho) on the Vitamin D Receptor (VDR) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *Cells were treated with vehicle (0.1% BSA) or 200 pM Klotho for 6, 24 or 48 hours. Results are presented as ratio of VDR protein expression and corresponding β-actin protein levels, as demonstrated by Western blot analyses. Results represent mean ± SEM, n = 6, * p < 0.05; vs. control; data were analysed by one-way ANOVA. Below the graph are the representative Western blots.*

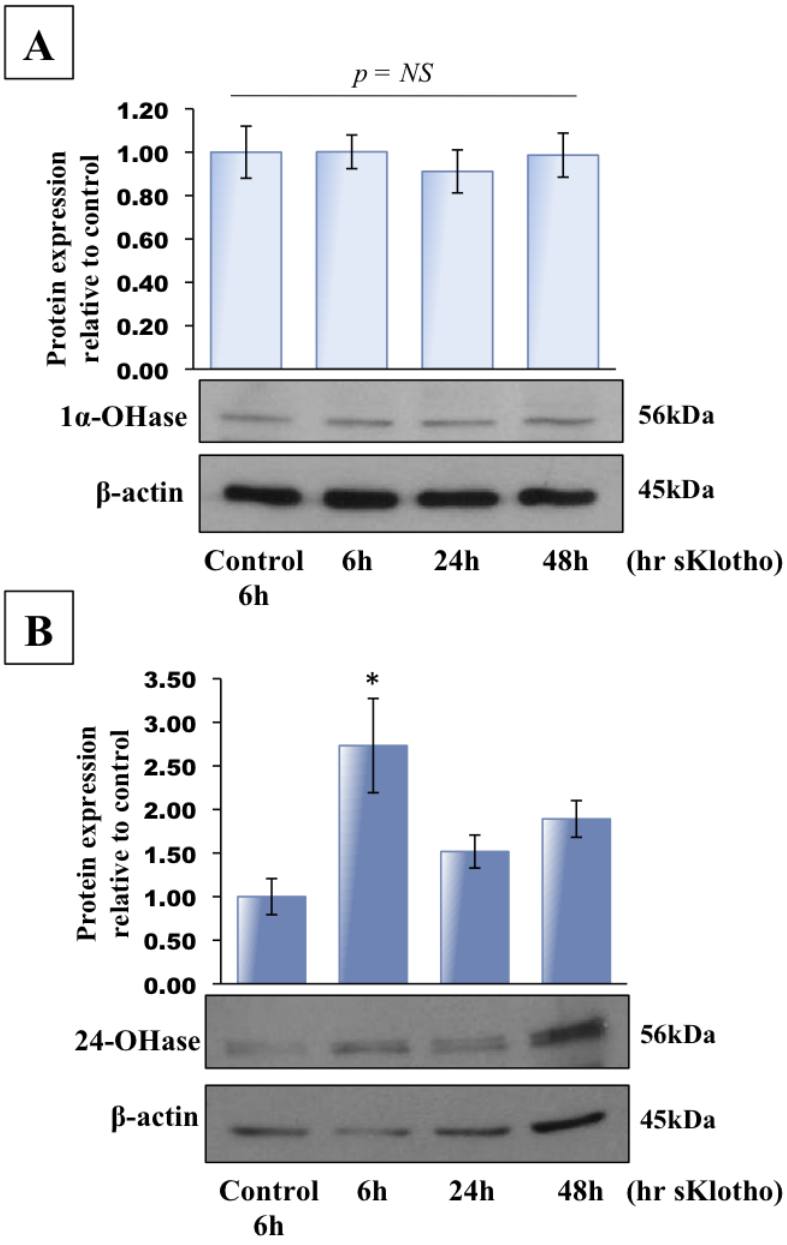


***Figure 6.9: The effect of human recombinant soluble Klotho (hr sKlotho) on the 1α-Hydroxylase (1α-OHase) and 24-Hydroxylase (24-OHase) protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) over time.*** *Cells were treated with vehicle (0.1% BSA) or 200 pM hr sKlotho with 10 μg/ml heparan sodium for 6, 24 or 48 hours. (A) 1α-OHase protein expression (B) 24-OHase protein expression, as demonstrated by Western blot analyses. Results are presented as ratio of (A) 1α-OHase or (B) 24-OHase protein expression and corresponding β-actin protein levels. Results represent mean ± SEM, n = 6, * p <0.05; vs. control; data were analysed by one-way ANOVA. Below each graph are the representative Western blots.*

### *6.2.2.2 sKlotho Potentially Prevents FGF Receptor from Being Blocked by Excess of FGF-23 Maintaining Stable mbKlotho Protein Levels*

In order to determine whether FCS influences the action of FGF-23 on mbKlotho protein expression in HAoSMCs, cells were grown and treated with FGF-23 (5 ng/ml or 100 ng/ml) in the presence or absence of FCS (0.5%) or in the presence of hr sKlotho, for 24 and 48 hours (treatment medium was renewed every 24 hours). In the absence or presence of 0.5% FCS in the medium, mbKlotho protein expression appeared to be inhibited by 100 ng/ml FGF-23 at 24 hours. However, in the absence of FCS, but in presence of hr sKlotho (200 pM) mbKlotho protein expression was unchanged (*Figure 6.10*). However, to fully prove this hypothesis further Western analyses and protein binding assays would be required.

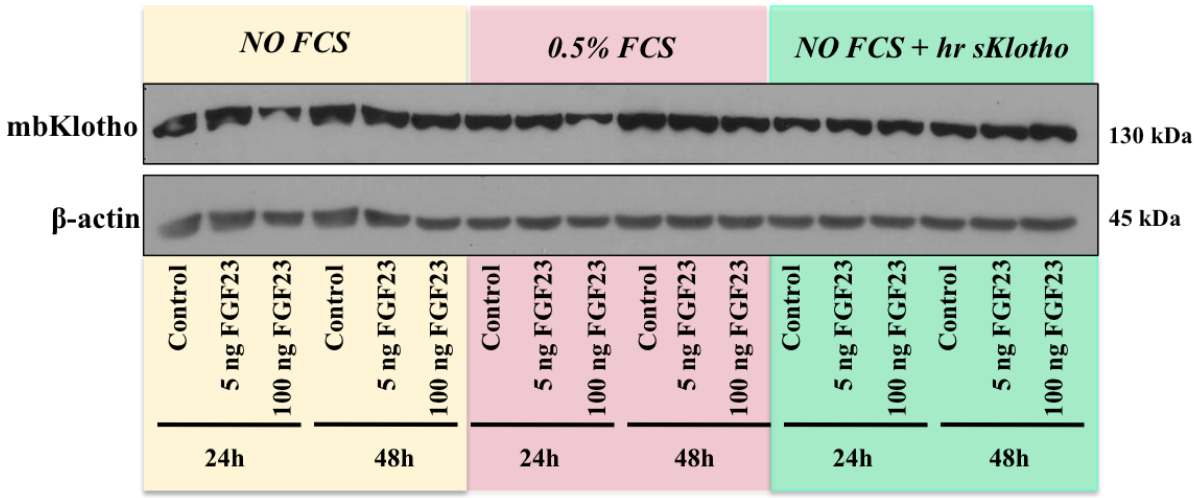


***Figure 6.10: The effect of the Fibroblast Growth Factor (FGF-23) on the Klotho protein expression in Human Aortic Smooth Muscle Cells (HAoSMCs) grown in absence or presence of Foetal Calf Serum (FCS) or in presence of human recombinant (hr) soluble Klotho.*** *Cells were treated with 5 ng/ml or 100 ng/ml FGF-23 for 24 and 48 hours and Klotho protein expression was assessed by Western blot analysis. Representative Western blots.*

## 6.3 Discussion

The results presented in this chapter show that VSMCs express the machinery required for the endocrine action of FGF-23; namely: FGFR1, FGFR3, mbKlotho, cKlotho and sKlotho. Further, my finding that the MEK-1 inhibitor, PD98059 effectively inhibited FGF-23-induced signalling confirmed that VSMCs respond to FGF-23. Previous studies have shown that that FGF-23 signal is also transduced via MAPK and phosphorylation of ERK1,2 in human chondrocytes and in human proximal tubule epithelial cells (Orfanidou *et al.* 2012, Perwad *et al.* 2007). An alternative approach to the one used in my experiments would involve neutralisation of FGF-23 with a specific anti-FGF-23 antibody or less directly, through Klotho knockout (Lim *et al.* 2012, Shalhoub *et al.* 2012).

My results demonstrated that FGF-23 modulates the local vitamin D system in VSMCs. FGF-23 significantly induced VDR protein expression at 24 hours. Significant 3-fold raise in 24-OHase protein was observed later, at 48 hours. Interestingly, the lower concentration of FGF-23 (5 ng/ml) had no effect on 1α-OHase protein expression. The ultimate consequence to VSMCs may be that FGF-23 makes cells more sensitive to $1,25(OH)_2D$. It appears that it may inhibit the VDR-1α-OHase-24-OHase system by upregulating 24-OHase; however enzyme activity data would aid the interpretation of these findings. The known positive regulators of bone derived FGF-23 are phosphate and $1,25(OH)_2D$. Evidence shows that both circulating $1,25(OH)_2D_3$ and the locally produced fraction can induce FGF-23 in primary differentiated osteoblasts (Tang W. J. *et al.* 2010). It is well known that FGF-23 acts on a variety of tissues. Data are currently emerging that highlight the importance of extra-renal $1,25(OH)_2D_3$ synthesis. These studies imply that FGF-23

can be an important player in regulation of the local and fine-tuning of the systemic VDR actions. Recent evidence from studies using chondrocytes suggests that the FGF-23 promoter contains two VDREs (Orfanidou *et al.* 2012).

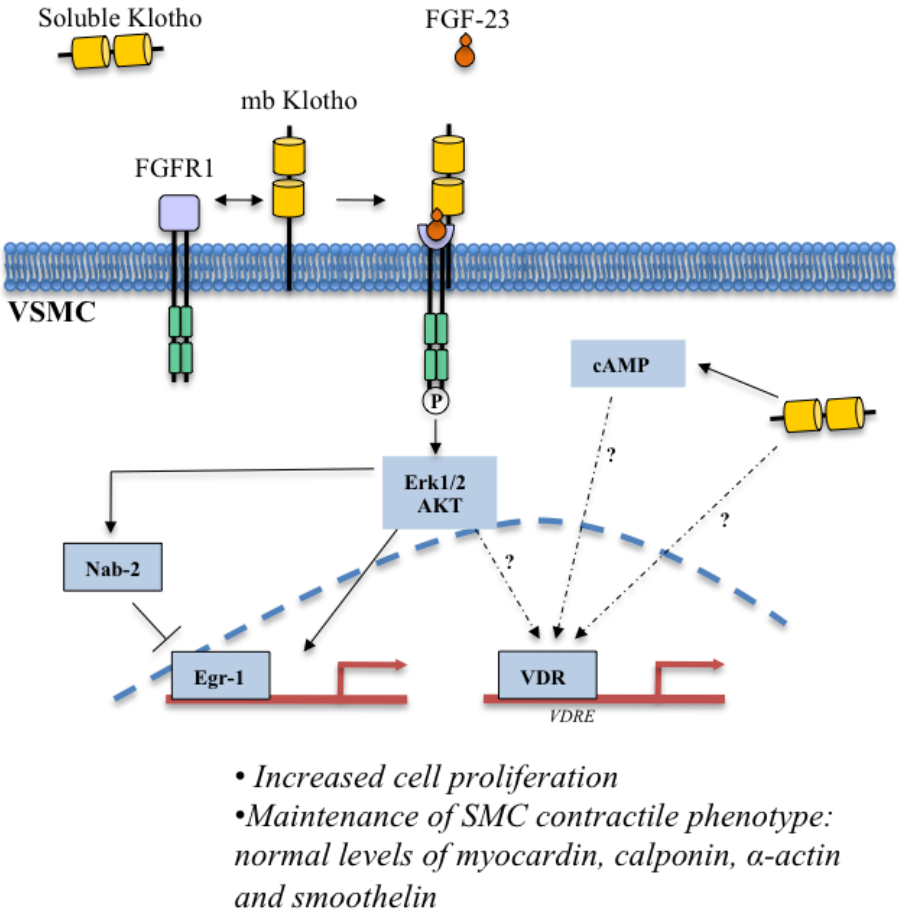


**Figure 6.11:** ***Schematic representation of Fibroblast Growth Factor 23 (FGF-23) and Klotho signalling in Vascular Smooth Muscle Cell (VSMC).*** *(mb)- membrane bound. (Arrow)-activation, (T-line) – inhibition, (double sided arrow) – complex formation, (?)- potential interaction.*

In human and mouse kidney proximal tubule cells, increasing doses of FGF-23, ranging from 1 to 10 ng/ml induced a dose dependent decrease in *CYP27B1* mRNA expression, with maximum suppression detected after 21 hours of treatment (Perwad *et al.* 2007). Similar effects of exogenous FGF-23 has been observed in monocytes from healthy and dialysis patients. Interestingly, suppression of *CYP27B1* was

accompanied by a decrease in *CYP24A1*, *FGFR1* and *Klotho* mRNA (Bacchetta J. Lisse, T. Hewison, M. 2011). An entirely opposite effect of FGF-23 has been observed in the parathyroid glands, where treatment with 100 ng/ml of FGF-23 (the same concentration as the one used in the monocytes' treatments) significantly upregulated *CYP27B1* mRNA after only 1 hour. Furthermore, FGF-23 regulation in chondrocytes demonstrated that despite FGF-23 being produced by osteoblasts or osteocytes, it is the VDR signalling in the chondrocytes that regulates the synthesis of this phosphatonin (Masuyama *et al.* 2006).

The FGF-23 inhibitory effect on 1α-OHase expression presented in this chapter is similar to the one observed in kidney and monocytes. Interestingly my results indicate that 1α-OHase protein suppression was only achieved in the presence of a high concentration of FGF-23 (100 ng/ml) and required the presence of 0.5% FCS. It is possible that either serum contains sufficient concentration of sKlotho for the added FGF-23 to act on VSMCs or that proteins present in the serum stabilised the FGF-23, which has a very short half-life, enhancing its biological potency *in vitro*. If the latter were true, one might speculate that the endogenous Klotho expressed by HAoSMCs facilitated the action of FGF-23. The combination of both hypotheses may also be plausible. Interestingly, our acute *in vitro* cell model mirrored what is observed clinically in CKD arteries: high serum concentration of FGF-23 and decreased 1α-OHase protein expression. Measurement of $1,25(OH)_2D$ levels post FGF-23 treatment in VSMC would be the next step to confirming whether FGF-23 has a direct inhibitory effect on $1,25(OH)_2D$ synthesis.

Klotho consistently induced VDR and 24-OHase protein expression by 6 hours of incubation, whereas no change in 1α-OHase protein was detected (similar observations were made in FGF-23 treatment). It appears that Klotho has an

independent effect on vascular vitamin D system, one that mirrors the effects of FGF-23. My results suggest that both Klotho and/or FGF-23 induce catabolism of $1,25(OH)_2D_3$ on a local level, as seen previously in kidney (Hu *et al.* 2010). My findings that Klotho independently induces 24-OHase protein expression support the observations made previously in Klotho-null mouse in which $1,25(OH)_2D$ levels are markedly elevated, despite high FGF-23 (opposite to what is observed in CKD, where serum $1,25(OH)_2D$ levels are low) demonstrating that high FGF-23 in absence of Klotho is insufficient to suppress the excessive $1,25(OH)_2D$ production (Dai *et al.* 2012, Dusso and Rodriguez 2012, Hu *et al.* 2011). Klotho is expressed on surface of cells forming new organs, upon cleavage it is released to circulation. Perhaps cleavage and consequently a size decrease facilitate the entry of Klotho into the cell allowing a direct interaction with VDR.

Experiments using *in vitro* VSMC showed that an increase in the local concentration of phosphate results in upregulation of 1α-OHase protein expression (Chapter 5). Further results presented in this chapter suggest that VSMCs have a mechanism through which they control phosphate homeostasis locally and that Klotho may be an important player, due to its ability to act on VDR in vasculature. Recent studies endorse my findings with the observation that soluble recombinant Klotho prevents phosphate induced VC in murine VSMCs through inhibiting the sodium-phosphate co-transporters Pit-1 and Pit-2 (Hu *et al.* 2012, Lau *et al.* 2012).

In conclusion, FGF-23 can act on VDR to exert different outcomes depending on the cell type; in vasculature it appears to act in a fashion different to kidney and macrophages, by suppressing 1α-OHase production only at extreme concentration and by controlling the system by the upregulation of 24-OHase. The interaction

between VDR and FGF-23 may be facilitated by cell-specific factors involved in the formation of the transcriptional complex. The fact that in the parathyroid cells FGF-23 stimulates 1α-OHase expression *in vitro*, exerting an action directly opposite to the one seen in kidney, bone and monocytes, may be an important element in the systemic FGF-23 regulation. Also, one may assume that increased FGF-23 contributes to vascular cytoprotection on two levels, first by inducing 1α-OHase associated immunoprotection and second, by partly contributing to the beneficial effects of calcitriol treatment in CKD. Although in the light of recent studies using a rat model, where specific neutralisation of FGF-23 in CKD rats led to increase in serum $1,25(OH)_2D_3$, calcium, and phosphate and a decrease in serum PTH levels, overall contributing to mortality, only highlights that a control of one factor may not necessarily be a best treatment option, ultimately raising many new questions (Shalhoub *et al.* 2012).

Data presented in this chapter support the existence of a vascular-endocrine FGF-23/Klotho axis and further provide insights into the complex regulation of mineral homeostasis within the vascular milieu.

# Chapter 7

# Conclusions and Future Directions

## 7.1 Key Findings

- VSMCs express functional Vitamin D System (VDR, 1α-OHase and 24-OHase mRNA and protein)

- Expression of the Vitamin D System is downregulated in CKD arteries compared with healthy arteries

- 25(OH)$D_3$ (100 nM) and 1,25$(OH)_2D_3$ (10 nM) independently largely induce *CYP24A1* mRNA; suppress *CYP27B1* mRNA and upregulate *VDR* mRNA in VSMCs; changes in mRNA are not mirrored by the changes in protein

- High calcium (2-3 mM) upregulates VDR expression and induces early and sustained increase in 1α-OHase and 24-OHase protein levels

- High phosphate (2-3 mM) does not affect the VDR protein expression, however it increases both the 1α-OHase and 24-OHase protein expression

- TNF-α strongly induces the *VDR* mRNA and protein expression in VSMCs and human artery. This induction is blunted in CKD arteries and partially reversed in presence of 1,25$(OH)_2D_3$

- VSMCs express the FGFR1, FGFR3 and respond to the FGF-23 via ERK1,2 phosphorylation pathway. Low dose of the FGF-23 (5ng/ml) induces the VDR and

24-OHase protein expression, whereas high dose (100ng/ml) suppresses 1α-OHase protein

- VSMCs express mbKlotho, sKlotho and cKlotho protein

- sKlotho mirrors FGF-23 by independently inducing the VDR and 24-OHase protein expression

- VSMC vitamin D system is regulated differently to the endocrine renal vitamin D system. It is also regulated differently to other previously described extra-renal vitamin D systems such as macrophages, parathyroid glands and bone (*Table 7.1*).

***Table 7.1 Regulation of renal and extra-renal 25(OH) - 1α-Hydroxylase protein expression.*** *Vascular Smooth Muscle Cell (VSMC), Fibroblast Growth Factor -23 (FGF-23), Interferon-γ (INF-γ), Tumour Necrosis Factor – α (TNF-α), Human Recombinant Soluble Klotho (hr sKlotho), low (0.2-0.5 mM), high (2-3 mM), *TNF-α: no effect on VSMC 1α-OHase, but induction of VDR, *hr sKlotho: no effect on VSMC 1α-OHase, but induction of Vitamin D Receptor and Vitamin D 24-Hydroxylase. Increase (↑), decrease (↓).*

| | 25(OH)D – 1α-Hydroxylase | | | | |
|---|---|---|---|---|---|
| | Kidney | Macrophages | Parathyroid | Bone | VSMC |
| **Calcium** | Low: ↑↑<br>High: ↓ | | High: ↑ | Low: ↑<br>High: ↑ | High: ↑ |
| **Phosphate** | Low: ↑↑<br>High: ↓ | | | High:↑ | High: ↑ |
| **$1,25(OH)_2D_3$** | ↓ | No effect | | No effect | ↓ |
| **INF-γ** | No effect | ↑ | | | ↑ |
| **TNF-α** | No effect | ↑ | | | No effect* |
| **FGF-23** | ↓ | ↓ | ↑ | ↓ | ↓ |
| **hr sKlotho** | | | | | No effect* |

## 7.2 Summarising Discussion

The experiments conducted for the purpose of this thesis increased the understanding of the complex vitamin D system. It is now established that there are multiple mechanisms through which $1,25(OH)_2D$ can modify cardiovascular outcomes. Its action can be exerted directly via VDR activation or, indirectly through regulation of calcium homeostasis. The key findings of this thesis were determination of expression of 24-OHase protein and mRNA in human arteries and primary cultures of HAoSMCs, followed by discovery that 24-OHase protein expression is elevated in arteries of patients with CKD, and 1α-OHase protein levels are decreased.

These changes may trigger abnormal responses to $1,25(OH)_2D_3$, especially by increasing 24-OHase synthesis. Consequently, from the clinical perspective, prolonged treatment with $1,25(OH)_2D_3$ and possibly other active vitamin D compounds may result in $1,25(OH)_2D$ resistance. This could be proven, by first examining whether the protein expression is reflected in enzyme activity then by clinical trial. Furthermore, extensive immunohistochemistry and Western blot analyses proved that altered expression of the VDR, 1α-OHase and 24-OHase in CKD arteries co-localised with medial calcification and increased levels of RUNX-2 and sclerostin. Recent clinical trials - seven longitudinal observational cohort studies and four randomised trials underline the importance of the described findings with the observation of no statistically significant effect of vitamin D supplementation on cardiovascular outcomes (Pittas *et al.* 2010). The available data are insufficient to postulate that cardiovascular outcomes can be corrected by increasing vitamin D intake or serum or plasma 25(OH)D levels. However according to the author of the

review analysing the clinical studies, the results may be skewed by chronic non-specific illness, smoking, alcohol or vitamin D fortified food intake.

Based on the results presented in this thesis it is not unreasonable to assume, that correction of vascular 1α-OHase and 24-OHase protein expression levels in CKD, without majorly affecting the circulating elements of vitamin D system and ultimately other hormones, could be achieved through local modulation of VSMCs with a combination of INF-γ, FGF-23 and TNF-α inhibitors. However in order to confirm that further *in vitro* studies using human arteries would have to be performed. Cell-specific method of administration would have to also be established. This method could then be compared with the use of new class of 24-OHase inhibitors (Chiellini *et al.* 2012).

## 7.3 Future Directions

Future work to confirm whether catabolic profile is in fact what induces $1,25(OH)_2D_3$ resistance in CKD arteries would broaden current perspective - a conditional vascular 24-OHase knock-out and over-expressing mice studies could prove helpful.

Continuation of cross-sectional analysis of arterial explants, reaching significantly higher number of samples could be useful to confirm the results presented in this thesis. Importantly, serum $25(OH)_2D_3$ levels should also be examined for each subject, ideally using most recent sensitive methods such as mass spectroscopy (Bruce *et al.* 2013). Furthermore, the selective therapeutic targeting of 24-OHase, 1α-OHase and VDR could deliver many answers. Recent advancement in the application and utilization of nanotechnology may aid the therapeutic stimulation of

1α-OHase or inhibition of 24-OHase enzymatic activity (Mai and Meng 2012, Park 2013). Lastly, establishment of simple and reliable method of measuring levels of substrate- $25(OH)D_3$ and product- $1,25(OH)_2D_3$ could even further improve our understanding of the importance of local synthesis of $1,25(OH)_2D_3$ in maintenance of healthy phenotype of VSMCs.

Despite our growing knowledge of VDR activation in CKD, we are still yet to fully understand the consequences of the auto/paracrine effects of vitamin D production. There is overwhelming evidence that its role in vasculature may be more complex - not only regulatory at the cellular level, but also systemic due to its fine-tuning actions of the endocrine system. Protective versus proactive effects of VDR activation in vasculature are a subject of extensive research to improve our current understanding of vascular calcification in CKD.

# Publications

1. *Vitamin D Signalling Inhibits the Development of an Osteoblastic Phenotype in Long-term Cultures of Arterial Explants.* Molostvov G, **Lubczańska MA**, Zehnder D, Bland R. 50th ERA-EDTA Congress, April 2013 MO030. Abstract.
2. *The Vascular Vitamin D Hormonal System Is Blunted In Arteries From Patients With CKD When Comparing To Arteries From Healthy Individuals.* Molostvov G, **Lubczańska MA**, Lam FT, Bland R, Zehnder. Renal Association UK, January 2013. Abstract.
3. *Expression of 25-Hydroxyvitamin $D_3$ 1-alpha Hydroxylase and Functional Vitamin D Signalling in Vascular Smooth Muscle Cells*. **Lubczańska MA,** Bland R, Zehnder D. Physiological Society Meeting, Edinburgh, December 2011. Oral Communication and Poster Presentation
4. *Modification of Human Arterial Inflammation by Paricalcitol is Altered in Chronic Kidney Disease*. Molostvov G, **Lubczańska MA**, Zehnder D. American Society of Nephrology Conference, Philadelphia, November 2011. Poster Presentation.

# Appendix

# Ethics Approval

SL14 Favourable opinion following consideration of further information
Version 2, October 2004

NHS

**Coventry Research Ethics Committee**
Trust Admin Centre 1
Walsgrave Hospital
Clifford Bridge Road
Coventry
CV2 2DX

26 May 2005

Dr Daniel Zehnder
Senior Lecturer / Honorary Consultant in Nephrology
University Hospital Coventry & Warwickshire
Department of Nephrology
Clifford Bridge Road
Coventry
CV2 2DX

Dear Dr Zehnder

***Full title of study:*** ***The expression of the extracellular calcium sensing receptor in human artery and its role in vascular calcification in patients with chronic kidney disease.***
***REC reference number:*** ***05/Q2802/26***
***Protocol number:***

Thank you for your letter of 10 May 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by Chairman.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

**Conditions of approval**

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| Document Type: | Version: | Dated: | Date Received: |
|---|---|---|---|
| Application | Version 1 | 17/02/2005 | 17/02/2005 |
| Investigator CV | Dr D Zehnder | 25/01/2005 | 17/02/2005 |
| Protocol | Version 3 | 12/02/2005 | 17/02/2005 |
| Statistician Comments | Yana Vinogradova | 12/02/2005 | 17/02/2005 |
| Letters of Invitation to Participants | Invitation Participants/Gp | 18/04/2005 | 20/04/2005 |

SL14 Favourable opinion following consideration of further information
Version 2, October 2004

| | | | |
|---|---|---|---|
| | Version 3 | | |
| GP/Consultant Information Sheets | Letter to GP/Consultant version 2 | 16/02/2005 | 17/02/2005 |
| Participant Information Sheet | Version 4 | 10/05/2005 | 17/05/2005 |
| Participant Consent Form | Version 4 | 10/05/2005 | 17/05/2005 |
| Response to Request for Further Information | Letter | 19/04/2005 | 20/04/2005 |
| Response to Request for Further Information | Letter Dr Zehnder | 10/05/2005 | 17/05/2005 |
| Other | Vascular Calcification Worksheet version 3 | 25/01/2005 | 17/02/2005 |
| Other | Letter from British Renal Society | 20/12/2004 | 17/02/2005 |

*Please note there is a typographical error in the Participant Information Sheet; Ms Wyman is the Complaints Manager for University Hospitals Coventry & Warwickshire NHS Trust. Please remember to alter before using these sheets.*

**Management approval**

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

**Notification of other bodies**

The Committee Administrator will notify the research sponsor and the R&D Department for NHS care organisation that the study has a favourable ethical opinion.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

**05/Q2802/26** **Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project,

Yours sincerely,

**Mrs C C Wright**
**Chairman**

E-mail: pauline.pittaway@uhcw.nhs.uk

*Enclosures* *Standard approval conditions*

*Site approval form (SF1)*

**Coventry Research Ethics Committee**

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

*For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.*

| | | | | | |
|---|---|---|---|---|---|
| **REC reference number:** | 05/Q2802/26 | **Issue number:** | 1 | **Date of issue:** | 26 May 2005 |
| **Chief Investigator:** | Dr Daniel Zehnder | | | | |
| **Full title of study:** | The expression of the extracellular calcium sensing receptor in human artery and its role in vascular calcification in patients with chronic kidney disease. | | | | |

*This study was given a favourable ethical opinion by Coventry Research Ethics Committee on 26 May 2005. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.*

Enclosure 3

| Principal Investigator | Post | Research site | Site assessor | Date of favourable opinion for this site | Notes [1] |
|---|---|---|---|---|---|
| Dr Daniel Zehnder | | University Hospitals Coventry and Warwickshire NHS Trust Cardiology Department | Coventry Research Ethics Committee | 26/05/2005 | |

Approved by the Chair on behalf of the REC:

..... (Signature of Administrator*)

(*delete as applicable)

..... (Name)

[1] *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

NHS
Central Office for Research Ethics Committees
(COREC)

## RESEARCH IN HUMAN SUBJECTS OTHER THAN CLINICAL TRIALS OF INVESTIGATIONAL MEDICINAL PRODUCTS

### Standard conditions of approval by Research Ethics Committees

1. Further communications with the Research Ethics Committee

1.1 Further communications during the research with the Research Ethics Committee that gave the favourable ethical opinion (hereafter referred to in this document as "the Committee") are the personal responsibility of the Chief Investigator.

2. Commencement of the research

2.1 It is assumed that the research will commence within 12 months of the date of the favourable ethical opinion.

2.2 In the case of research requiring site-specific assessment (SSA) the research may not commence at any site until the Committee has notified the Chief Investigator that the favourable ethical opinion is extended to the site.

2.3 The research may not commence at any NHS site until the local Principal Investigator (PI) or research collaborator has obtained management approval from the relevant NHS care organisation.

2.4 Should the research not commence within 12 months, the Chief Investigator should give a written explanation for the delay. It is open to the Committee to allow a further period of 12 months within which the research must commence.

2.5 Should the research not commence within 24 months, the favourable opinion will be suspended and the application would need to be re-submitted for ethical review.

3. Duration of ethical approval

3.1 The favourable ethical opinion for the research applies for the expected duration of the research as specified in the application form. If it is proposed to extend the duration of the study, this should be submitted for approval as a substantial amendment.

4. Progress reports

4.1 Research Ethics Committees are required to monitor research with a favourable opinion. The Chief Investigator should submit a progress report to the Committee 12 months after the date on which the favourable opinion was given. Annual progress reports should be submitted thereafter.

4.2 Progress reports should be in the format prescribed by COREC and published on the website (see www.corec.org.uk).

4.3 The Chief Investigator may be requested to attend a meeting of the Committee or Sub-Committee to discuss the progress of the research.

5. Amendments

5.1 If it is proposed to make a substantial amendment to the research, the Chief Investigator should submit a notice of amendment to the Committee.

5.2 A substantial amendment is any amendment to the terms of the application for ethical review, or to the protocol or other supporting documentation approved by the Committee, that is likely to affect to a significant degree:

(a) the safety or physical or mental integrity of the trial participants

(b) the scientific value of the trial

(c) the conduct or management of the trial.

5.3 Notices of amendment should be in the format prescribed by COREC and published on the website, and should be personally signed by the Chief Investigator.

5.4 A substantial amendment should not be implemented until a favourable ethical opinion has been given by the Committee, unless the changes to the research are urgent safety measures (see section 7). The Committee is required to give an opinion within 35 days of the date of receiving a valid notice of amendment.

5.5 Amendments that are not substantial amendments ("minor amendments") may be made at any time and do not need to be notified to the Committee.

6. Changes to sites *(studies requiring site-specific assessment only)*

6.1 Where it is proposed to include a new site in the research, there is no requirement to submit a notice of amendment form to the Committee. Part C of the application form together with the local Principal Investigator's CV should be submitted to the relevant LREC for site-specific assessment (SSA).

6.2 Similarly, where it is proposed to make important changes in the management of a site (in particular, the appointment of a new PI), a notice of amendment form is not required. A revised Part C for the site (together with the CV for the new PI if applicable) should be submitted to the relevant LREC for SSA.

## Working with Human Samples
## Registration of individual researchers

## University of Warwick

The University of Warwick maintains a register of all researchers working with human samples. Registration requires the researcher to undertake training appropriate to their research needs and to maintain a training programme that demonstrates they are competent to perform duties appropriate to their role in each research project. The responsibility for ongoing personal development rests with the individual researcher.

**Declaration of Registration**
I believe that I have received adequate information, instruction and training to be able to carry out my work with human tissue safely and in accordance with the Human Tissue Act (2004) and the University's Standard Operating Procedures. I will at all times follow the appropriate instructions I have been given and adopt safe working practices.

I have read/attended and understood the following documents/presentations:

- HTA Code of Practice 1 – Consent
- HTA Code of Practice 9 – Research
- Briefing Session 1 (Knowing your Responsibilities)
- MRC e-learning module (www.rsclearn.mrc.ac.uk)

In the event of any situation arising where I am not sure about the appropriate action to take I will seek advice before proceeding. Where appropriate, I will bring to the attention of my supervisor and/or Lead Investigator for the research project any concerns that I have in relation to my work with human samples. If I still have concerns, or where I am Lead Investigator or Person Responsible, I will notify the Designated Individual (or their named representative).

Name: MARIA LUBCZANSKA

Signed: _____ Date: 31.08.2010

**Lead Investigator or Person Responsible (where applicable)**
I confirm that I accept overall responsibility for the involvement of the above named researcher on research projects involving human samples that I am custodian for.

Name: DR DANIEL ZEHNDER

Signed: _____ Date: 31/8/10

**Confirmation of Registration**
I confirm that the above named researcher is registered for working with human samples at the University of Warwick.

Name: John Davey (Designated Individual, HTA Licence #12297)

Signed: J. Davey Date: 2 SEPTEMBER 2010

**All researchers working with human samples are required to update their skills at least every 3 years. This registration will be due for renewal on** SEPTEMBER 2013

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